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(54) Title: MARKER FOR IDENTIFYING HEMATOPOIETIC STEM CELLS

(57) Abstract: The present invention is related to a method for identifying, isolating and manufacturing pluripotent hematopoietic stem useful as transplants for treating blood diseases. The hematopoietic stem cells and/or committed progenitor cells are identified using antibodies capable of specifically recognizing human podocalyxin- like proteins (hPCLPs) expressed in hematopoietic tissues. The : human hematopoietic tissue derived hhPCLPs are useful as markers for assessing the quality of stem cells obtained by conventional methods and which comprise only a fraction of undifferentiated pluripotent stem cells. The hhPCLP differs from other known PCLPs in that it is obtainable from the hematopoietic tissue, has a specific glycosylation pattern and in its glycosylated form an apparent MW of lOO-llO kDa. Said hhPCLP can be identified with antibodies raised against PCLPs from different species and tis- sues by per se known methods.

MARKER FOR IDENTIFYING HEMATOPOIETIC STEM CELLS

The Technical Field of the Invention

The present invention is related to a novel human hematopoietic tissue derived podocalyxin-like protein (hhPCLP) useful as a cell surface marker for identifying, purifying, isolating, separating and producing hematopoietic stem cells as well as such isolated and purified hematopoietic stem cells which are useful as transplants or grafts for treating severe blood diseases, deficiencies or damages in the blood forming tissues. The present invention is also related to the use of podocalyxin-like protein (PCLP), especially, the extracellular parts of PCLP and most particularly the extracellular parts of hhPCLP for manufacturing binding substances capable of specifically recognizing said marker on stem cells.

The Background of the Invention

Originally bone marrow cells have been used for treating severe blood diseases, but for the last ten years the use of stem cells mobilized in peripheral blood has increased, even if most of the cells in peripheral blood are mature cells or at least partly differentiated. CD34 positive stem cell transplants derived from mobilized peripheral blood have been used for treating patients suffering from severe blood diseases, including leukemia, lymphoma, immune deficiency and/or other diseases, the treatment of which, including radio- and chemotherapy, causes severe and/or permanent damages in their blood cell renewal system. The stem cell and/or committed progenitor cell transplants used are generally autologic. In other words, they are derived from the patients and are transferred back to the donors themselves after the radio- or chemotherapy. Alternatively, allogenic transplants can be used. In said case the CD34 positive stem cells are obtained from mobilized peripheral blood or from placental blood. Especially, the latter is known for seldom causing rejection of transplants and thus being well tolerated by the patient.

As said above most of the cells in blood are differentiated mature blood cells. Even after mobilization, i.e. treatment with

growth factors only about 1 % of the leukocytes in the peripheral blood are CD34 positive. At present the surface antigen CD34 is considered to be one of the most useful markers from stem cells.

This means that about 99 % of the leukocytes are of no use as stem cells. Said partly differentiated progenitor cells comprise different kinds of cells at various stages of differentiation and can be identified and roughly classified with known stem cell markers. Peripheral blood leukocytes devoid of stem cells can be useful in some situations e.g. in transfusions given to immunodeficient patients, when there might be a danger of a graft vs. host response. Leukocytes, which are preidentified with known stem cell markers, and which are not pluripotent stem cells and thus unapplicable for grafting, include several partly differentiated cell types, which could provide useful material or reagents for test kits or for studying the differentiation of stem cells.

Among the cell population comprising about 1 % of mobilized CD34 positive progenitor cells only a minor fraction, in fact about 15 % are so called pluripotent stem cells, which have the capacity of colony formation with the colony formation or CFU/BFU-assay described by Ema et al. (Blood 75:1941-1946, 1990), which indicates the capacity of stem cells to start new blood production. Because cell cultivation is a rather tedious and time-requiring process, taking up to two weeks, it would be advantageous, if said 15 % undifferentiated pluripotent stem cells could be identified using more rapid methods. In order to improve and make the identification of pluripotent stem cells more complete and/or diversified, the CD34 marker is often used in combination with other markers. In order to avoid the need of additional identification steps and to avoid discarding cells, potentially useful for other purposes, it is evident that alternative and/or better markers with characteristics complementary to previously used markers would be advantageous.

A clear demand exists for providing new alternative markers, which as such or in combination with previously known markers have the capacity of identifying, isolating and manufacturing pluripotent stem cells and/or committed progenitor cells and to

enable assessment of the quality of the stem cells, i.e. determine the amount of pluripotent stem cells in a preidentified isolated stem cell population.

An ideal stem cell marker should fulfill certain prerequisites. It should not be expressed by fully differentiated cells, such as mature platelets, lymphocytes, monocytes, granulocytes and/or megakaryocytes. The ideal markers should be specific only for pluri-, multi- or totipotent hematopoietic stem cells and/or more or less committed stem cells. They should preferably be able to identify hematopoietic stem cells directly without using the colony formation assay. So far no such marker is known, and only different combinations of known antigens, usually CD34 combined with CD34+38-, Thy-1(CD90), c-kit (CD117) and/or other cell surface markers, have been used in attempts to distinguish the actual colony forming cells from the rest of the more developed partly differentiated committed progenitor cells.

From the discussion above it is evident that human hematopoiesis is still poorly understood and alternative markers for studying hematopoiesis would be urgently needed as well as such markers, which are capable of identifying pluripotent cells, capable of forming all different kinds of blood cells. It would also be advantageous to have markers capable of differentiating pluripotent stem cells from those cells, which already have differentiated to some extent including committed progenitor cells such as myeloid and/or lymphoid stem cells as well as colony forming CFU/BFU cells. Those cells which cannot be used as stem cells or committed progenitor cells could be useful for other purposes, e.g. as reagents in test kits useful for studying stem cell differentiation. Another useful application might be transfusion of committed stem or progenitor cells, i.e. cells capable of differentiating into only one or some lineages. This might be desirable in some special situations such as temporary bone marrow suppression.

Podocalyxin-like protein (PCLP) is a member of the sialomucin protein family sharing structural features and to some extent sequence homology with the CD34 protein and endoglycan. Previously, PCLP has been demonstrated only on the apical surface of renal

podocytes and endothelial cells in humans and in some other species (Kerjaschki, et al., J Cell Biol, 98:1591-1596, 1984; Kerjaschki, et al., J Clin Invest, 78:1142-1149, 1986; Kershaw, et al., J Biol Chem, 270:29439-29446, 1995).

For example, a mouse PCLP was recently described to be present in hemangioblasts, the early hematopoietic and endothelial cell progenitors (Hara, T., et al., Immunity 11: 567-578, 1999). However, no details about PCLP expression at later developmental stages or in adult mice have been reported. Results obtained with adult rat, murine rodent, show that podocalyxin is present in megakaryocytes and mature platelets. The presence ofpluripotent stem cells have not been demonstrated in rats. Said results indicate that PCLP would not be applicable as a specific marker for identifying pluripotent hematopoietic stem cells.

Sassetti, et al. (Journal of Experimental Medicine, 187:1965-1975, 1998), has described a human PCLP expressed on high endothelial venules of peripheral lymphoid organs carrying a MECA79 epitope which acts as a ligand for leukocyte L-selectin, which ligand is not found in PCLPs expressed by other tissues. A similar selectin-binding podocalyxin-like protein with a molecular weight of about 60-65 kDa based on its amino acid sequence, has been described in the International patent application WO 99/41363. Said PCLP protein has been isolated from endothelial cells of mammalian lymphoid organs. The protein itself, its nucleotide sequences and antibodies are disclosed and simultaneously their applicability for research, diagnostic and therapeutic applications, especially for treating infectious diseases. The document is totally silent about the presence of the molecule in leukocytes or the potential use of PCLP as a marker for stem cells.

The findings that PCLP is expressed by human hematopoietic tissue derived stem cells, enables the development of methods and means including selection of suitable binding substances and reagents for identifying, purifying and/or isolating pluripotent hematopoietic stem cells and for separating them from partially differentiated stem cells and for assessing the amount of pluripotent stem cells in mobilized stem cell populations. In other words,

the value of a preidentified stem cell population can be assessed and it is possible to distinguish the valuable pluripotent stem cells from other, partially differentiated cells, which can be isolated and used for other specific purposes, including transfusions given to immunodeficient patients, when there might be a danger of a graft vs. host response or as temporary bone marrow syndrome.

Accordingly, the objective of the present invention is to provide an alternative marker for more effective identification and isolation of hematopoietic stem cells useful as transplants for treating patient suffering from induced or spontaneous bone marrow dysfunctions.

Another objective of the present invention is to use podocalyxin-like protein (PCLP), especially its extracellular domains, and more particularly human hematopoietic podocalyxin-like protein (hhPCLP) for manufacturing binding substances, including antibodies and/or ligands useful for identifying, purifying and/or isolating stem cells and for differentiating them from other partially differentiated stem cells in a preidentified stem cell population.

Still another objective of the present invention is to provide nucleotide sequences which are capable of hybridizing with hhPCLP mRNA expressed by hematopoietic stem cells and which are useful as probes and/or primers.

The objective of the present invention is also to distinguish and recover those cells, which are not applicable as pluripotent hematopoietic stem cells and to use them for immunosuppression and for providing test kits for studying stem cell differentiation.

Isolated and purified hematopoietic stem cells carrying said hhPCLP marker are also the object of the present invention.

The Summary of the Invention

In the present invention, it is for the first time shown that a new type of podocalyxin-like protein (PCLP) is expressed in human

colony forming hematopoietic stem cells especially on their cell surface. These results indicate that said human hematopoietic cell derived podocalyxin-like protein (hhPCLP) is a new valuable marker for identifying hematopoietic stem cells.

The present invention is related to a hematopoietic tissue derived human podocalyxin-like protein (hhPCLP) having properties distinguishing it from other human podocalyxin-like proteins of other tissues. Said hhPCLP is useful as a marker for identifying, purifying and isolating hematopoietic stem cells and for manufacturing such cells for treating blood diseases by grafting.

Said hhPCLP is expressed by human hematopoietic tissues and has in its glycosylated form an apparent MW of approximately 100-110 kDa as calculated from sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE). hhPCLP has a specific glycosylation pattern distinguishing it from PCLPs expressed by other non-hematopoietic tissues in humans and/or other species. The glycosylated forms of human glomerular and/or endothelial tissue derived PCLPs are characterized by molecular weights of 140 kDa as well as 155/165 kDa depending upon the method of determination. The hhPCLP of the present invention expressed by hematopoietic stem cells is (specifically) recognizable by binding substances, e.g. antibodies and/or ligands, which are capable of recognizing the extracellular parts or domains of PCLP. Some useful binding substances, especially some antibodies, including monoclonal and/or polyclonal antibodies, which are capable of recognizing hhPCLP in lysates from hematopoietic cells are available from different sources. Other more useful binding substances capable of recognizing the extracellular domains of hhPCLP on intact hematopoietic stem cells can be prepared using per se known methods for raising antibodies including raising polyclonal antibodies and/or hybridoma techniques. Antibodies recognizing the specific glycosylated domains of hhPCLP expressed by hematopoietic tissue are particularly advantageous. An example of such an antibody, being specific for hhPCLP, was deposited on 21 May 2002 at the DSM2 Braunschweig, Germany (DI BB8 BB11 with the deposition No.....). This antibody has been made against K-562 cells and stains (by immunofluorescence (IF)) the membrane of K-562 cells, but does not stain glomeruli or endothelial cells by

IF. It binds to podocalyxin extrapeptide by ELISA and to GST (glutathione transferase)-PCLP extradomain fusion protein by immunoblotting. It binds to a 100-110 kD band in CFU cells, in K-562 cells and in glumeruli by immunoblotting. Thus, it binds PCLP peptide and stains hematopoietic cells, but does not bind to glomeruli or endothelia at tissue sections. It is therefore clear that D1 BB8 BB11 ("BB8") is functionally specific for hhPCLP.

The present invention is accordingly related to the use of human podocalyxin (hPCLP), especially hhPCLP, useful as a marker for stem cells and for preparing binding substances including antibodies, ligands and parts thereof, especially raising antibodies having the capacity of specifically recognizing hhPCLP on intact hematopoietic stem cells. Said marker and binding substances are especially useful for identifying hematopoietic stem cells.

Antibodies, recognizing extracellular domains of PCLP could advantageously be used for identifying, purifying and/or isolating pluripotent hematopoietic stem cells and/or committed progenitor cells from blood products, including peripheral blood, but also for differentiating between more or less differentiated cell lines e.g. from preidentified stem cell populations, which have been preidentified, with cell markers such as CD34, CD38, CD90, CD117 and/or other cell surface antigens.

The present invention is also related to a method for identifying, purifying and/or isolating hematopoietic stem cells from blood products including bone marrow, placental blood and/or mobilized or non-mobilized peripheral blood. Said hematopoietic stem cells are useful for providing tissue transplants or grafts for patients suffering from severe blood diseases, including immune deficiency, leukemia, lymphoma or damages caused for example by radio-, chemo- or immunotherapy.

The identification, purification and/or isolation methods of the present invention are characterized in that the blood product is contacted with a binding substance, for example an antibody, ligand or parts thereof capable of specifically recognizing an epitope in or on the extracellular part of human hematopoietic tissue derived podocalyxin-like protein (hhPCLP). The binding

substance, especially the antibody can be used as such for recognizing the marker, but for identifying, isolating, purifying and/or screening purposes, the binding substances, antibodies or alternatively the cells to be recognized are conveniently attached to a solid carrier, examples of which include chromatographic columns, magnetic beads, latex particles, microparticles, slides, tubes, plates, microwells, pegs, etc.

Binding substances or antibodies capable of binding to the hhPCLP marker can be made recordable by the aid of label-carrying marker molecules (tracers), especially fluorescent labels including fluorochromes. These labels can be used for recognizing PCLP-positivecells by various per se known techniques. The most preferred techniques are magnetic purification columns or fluorescence assisted cell sorting (FACS) techniques, which facilitate further isolation of the cells.

The present invention is also related to a method for manufacturing pluripotent hematopoietic stem cells useful for the treatment of blood diseases by transplantation. In the manufacturing or production method hematopoietic stem cells identified with the binding substances, antibodies, ligands or parts thereof, capable of specifically recognizing or binding to hhPCLP present on the surface of potential stem cells are subsequently isolated and optionally cultivated by per se known cell culture methods.

The present invention also relates to a preparation or culture of pluripotent hematopoietic stem cells wherein more than 20% of all cells are pluripotent hematopoietic stem cells and 80% or less are other cells (e.g. cells which are otherwise responsible for adverse reactions when administered to a patient (e.g. graft vs. host-reactions). Preferably, the preparation or culture according to the present invention comprises more than 50, more preferred more than 70, especially more than 90%, pluripotent hematopoietic stem cells. With the present invention it is even possible to provide cultures of such cells which comprise more than 95%, preferably more than 98%, even up to 100%, pluripotent hematopoietic stem cells. Such cultures or preparations of cells according to the present invention are preferably comprising more than 20% of cells being hhPCLP$^+$, more preferred more than 30%,

especially more than 50%, hhPCLP$^+$ cells. In the early stage of cloning such cells, preparations or cultures having more than 70%, preferably more than 90%, of hhPCLP$^+$ cells are provided by the present invention.

The present invention is also related to a method for treating blood diseases. The subjects or patients in need are provided with a transplant or graft comprising isolated hematopoietic stem cells separated from the subjects or patients themselves before radiation or cytostatic treatment. The stem cells are identified by the method of the present invention using antibodies recognizing extracellular parts of hhPCLP on the cell surface and the isolated cells are multiplied by optional per se known cell cultivation methods and the patient or donors are provided with an effective tolerable amount of the stem cells after radiation or cytostatic treatment.

The present invention also provides a method for identification of malignant hematopoietic cells, in some leukemia, lymphoma or other blood disorders.

The present invention also provides a method for differentiating pluripotent hematopoietic stem cells from other partially differentiated less primitive cells useful for providing other potentially useful blood products. In said method hematopoietic stem cells are separated from other more differentiated cells by contacting the blood product, including, bone marrow, mobilized or non-mobilized peripheral blood, placental blood, potential preidentified stem cell populations isolated by the aid of a first known stem cell marker and its binding substance and contacting said preidentified stem cell population with a second antibody capable of specifically binding to and recognizing an epitope preferably on the extracellular part of podocalyxin-like protein (PCLP or an intact hematopoietic stem cell). The recognized stem cells are thereafter recovered for providing suitable transplants. The preidentified stem cells, which are not binding to the binding substances, preferably antibodies recognizing the extracellular domains of PCLP, especially hhPCLP are also recovered or collected and used for providing valuable reagents for studying for example stem cell differentiation and for immunosuppres-

sion as described above.

A method for assessing the quality of preidentified stem cell population is also provided by the present invention. In said method an isolated stem cell population preidentified as carrying a stem cell surface antigen marker is contacted with a binding substance, such as an antibody capable of specifically recognizing the extracellular domains of hhPCLP and the hhPCLP positive cells are counted by known methods, such as FACS analysis.

The characteristic features of the invention are otherwise as defined in the claims.

Abbreviations

Ab	antibody
BFU	burst-forming unit
BFU-E	burst-forming unit-erythroid
CD34+	a CD34 positive cell surface antigen
CFU	colony-forming unit
CFU-E	colony-forming unit-erythroid
CFU-Eo	colony-forming unit-eosinophil
CFU-G	colony-forming unit-granulocyte
CFU-GM	colony-forming unit-granulocyte-monocyte
CFU-M	colony-forming unit-monocyte
CFU-MEG	colony-forming unit-platelet
FACS	fluorescence assisted cell sorting
FITC	fluorescein isothiocyanate
hPCLP	human podocalyxin-like protein
hhPCLP	human hematopoietic podocalyxin-like protein
HU-VEC-C	endothelial cells
kDa	kiloDalton
mAb	monoclonal antibody
MW	apparent molecular weight or radial molecular weight as determined by SDS-PAGE.
OSGE	O-sialoglycoprotein
PCLP	podocalyxin-like protein

A Short Description of the Drawings

Figure 1 is a schematic drawing of the hPCLP mRNA. The figure illustrates the location of the primers on both sides of the transmembrane region of the corresponding hPCLP protein. The numbers refer to the respective base pairs (bp) according to the GenBank™ accession number U97519. The double-ended arrow illustrates the area coding for the 19 amino acid C-terminal intracellular peptide used for immunization.

Figure 2a shows hPCLP in committed hematopoietic stem cells. The immunofluorescence (IF) micrograph of BFU-E colony derived cells shows positive staining with affinity purified anti-intracellular hPCLP peptide antibodies (#014). Magnification 400 x.

Figure 2b shows hPCLP in committed hematopoietic stem cells. The immunofluorescence micrograph of BFU-E colony derived cells shows positive staining with anti-glomerular hPCLP mAb 1B6. Magnification 400 x.

Figure 2c shows hPCLP in committed hematopoietic stem cells. The immunofluorescence micrograph of BFU-E colony derived cells shows no staining of the cells with anti-glomerular hPCLP mAb GC7. Magnification 400 x.

Figure 2d shows hPCLP in committed hematopoietic stem cells. The immunofluorescence micrograph of BFU-E colony derived cells shows no staining with normal rabbit IgG (negative ab control). Magnification 400 x.

Figure 2e shows hPCLP in committed hematopoietic stem cells. The immunofluorescence micrograph of CFU-M colony derived cells shows positive staining with #014. Magnification 400 x.

Figure 2f shows hPCLP in committed hematopoietic stem cells. The immunofluorescence micrograph of CFU-Meg colony derived cells shows positive staining with #014. Note that the positive cells are not yet polyploid. Magnification 400 x.

Figure 2g shows hPCLP in committed hematopoietic stem cells. The immunofluorescence micrograph of CD34+ cells shows positive staining with #014. Magnification 400 x.

Figure 2h shows hPCLP in committed hematopoietic stem cells. The immunofluorescence micrograph of K526 cells also shows positive staining with #014. Magnification 400 x.

Figure 2i shows hPCLP in human kidney. All anti-hPCLP antibodies stained glomeruli and endothelia at cryostat sections of human kidney as illustrated in the immunofluorescence micrograph demonstrating staining with mAb GC7. Magnification 100 x.

Figure 2j shows hPCLP in human kidney. All anti-hPCLP antibodies stained glomeruli and endothelia at cryostat sections of human kidney as illustrated in the immunofluorescence micrograph demonstrating staining with #014. Magnification 25 x.

Figure 2k shows hPCLP in cultured HU-VEC-C endothelial cells. The intracellular binding specificity of #014 is demonstrated in the immunofluorescence micrograph by positive staining of HU-VEC-C cells fixed with permeabilising acetone. Magnification 275 x.

Figure 2l shows that the intracellular binding specificity of #014 is demonstrated as no binding of antibodies in cultured HU-VEC-C endothelial cells fixed with paraformaldehyde. Paraformaldehyde stabilizes the cell membrane so that the intracellular parts are not available for the antibodies. Magnification 275 x.

Figure 3a shows characterization of the glomerular hPCLP antigens in glomerular (GLOM) lysates by Western blotting. #014 Abs and the mAbs GC7 and 1B6 recognize the same 155/165 kDa doublet in the glomerular (GLOM) lysates (- lanes). After treatment of the lysates with O-sialoglycoprotein endopeptidase enzyme (OSGE) (+ lanes), all Abs recognize remaining original high MW hPCLPs and the new, partially degraded high MW products, but only #014 Abs recognize the small products presumably devoid of the mucin domain.

Figure 3b shows characterization of the glomerular and endothelial hPCLP antigens in HU-VEC-C lysates. In HU-VEC-C lysates (- lanes) #014 recognizes mainly a 63 kDa protein and only weakly the high MW doublet (barely recognizable), whereas mAb GC7 gives

a strong reaction with the 155/165 kDa doublet, but does not react with the 63 kDa protein. After OSGE treatment of HU-VEC-C lysates (+ lanes) #014 reacts with the small MW degradation products, while all specific reactivity of mAb GC7 is lost.
Figure 3c shows that both the high and low MW hPCLPs detected in HU-VEC-C lysates (- lanes) are terminally N-glycosylated mature proteins, as treatment with Endo-F enzyme affecting only the terminally N-glycosylated proteins (+ lanes) changes the mobility of these proteins both in glomerular lysates (arrows) and in the HU-VEC-C lysates.

Figure 3d shows that both the high (arrow) and low hPCLPs of HU-VEC-C lysates (- lanes) are resistant to treatment with Endo-H enzyme (+ lanes) affecting only the non-terminally glycosylated immature N-glycosylated proteins. Thus both molecular forms of hPCLP represent mature glycoproteins.

Figure 4 shows that all anti-hPCLP antibodies recognize the same glomerular protein as shown by immunoprecipitation. By Western blotting, both the anti-peptide #014 Abs and mAb 1B6 recognize the 155/165 kDa protein doublet in the immunoprecipitates (Precip) obtained from the glomerular lysates (Norm) with mAb GC7. #014 detects the doublet also in the immunoprecipitate obtained from the HU-VEC-C lysates. Note that mAb GC7 does not precipitate the 63 kDa major antigen detected by #014 in the HU-VEC-C lysate. In the glomerular lysate (GLOM) #014 binds to an approximately 100 kDa protein not precipitated by mAb GC7. This reaction was seen also with the control Abs (not shown) and is probably non-specific.

Figure 5a shows that CFU- and BFU-staged hematopoietic stem cells express small forms of hPCLP. Left panel: #014 Abs bind to a 63/55 kDa doublet in the lysates of the CFU-GM and BFU-E staged cultured bone marrow cells, as shown by Western blot. The major bands detected in the lysates of mobilized peripheral blood CD34+ cells are ca. 50 and 90 kDa. CFU-GM mix contains CFU-G, CFU-M and CFU-GM colony cells. Glomerular and HU-VEC-C cell lysates were used as controls. The reactions are specific as preincubation of #014 with the immunogen peptide (right panel), but not with a control peptide (left panel) inhibits binding.

Figure 5b shows that CFU- and BFU-staged hematopoietic stem cells express small forms of PCLP. Also mAb 1B6 recognizes the 63 kDa protein from the lysates of all CFU- and BFU-staged cells, while mAb GC7 does not. mAb binds to a 119 kDa protein in all lysates except the CFU-G lysate. Normal mouse IgG was used as control.

Figure 6a depicts hPCLP mRNA in committed hematopoietic stem cells by nested RT-PCR. The size of the cDNA product obtained with the inner primers is 268 bp. hPCLP mRNA is detected in hematopoietic CFU-colony derived stem cells [BFU-E (2), CFU-G (3), CFU-M (4), CFU-GM (5), CFU-Meg cells (6)]. hPCLP was detected also in CD34+ cells isolated from mobilized peripheral blood (7), and K562 leukemia cells (8). Human glomerular mRNA (1) was used as positive control.

Figure 6b depicts hPCLP mRNA in hematopoietic stem cells and in mature leukocytes by nested RT-PCR. The size of the cDNA product obtained with the inner primers is 268 bp. hPCLP mRNA is detected in peripheral blood T-lymphocytes (1), B-lymphocytes (2), monocytes (3), and granulocytes (4), but not in erythrocytes (5) or thrombocytes (6). The ß-actin controls show that the isolation of RNA was successful.

Figure 7 shows comparison of the mRNA species expressed in mobilized CD34+ cells, renal cortex, and endothelial cells by Northern blotting. The 5.4 kb mRNA is the major form in all these cell types. Minor bands of 8 and 4.2 kb could also be detected. The expression level of CD34+ cells is low: more RNA was loaded, as shown by the ß-actin lanes, and a four times longer exposure time was needed.

Figure 8a shows PCLP antigens in mobilized CD34+ cells, K562 leukemia cells and detached HU-VEC-C cells by FACS analysis. Of the CD34+ cells of the mobilized peripheral blood, 15 % are positive for hPCLP, as shown by staining with the FITC-conjugated affinity purified anti-hPCLP antibodies (#015, grey areas). Also 75 % of the K562 cells were positive. As negative controls three different purified rabbit antibody controls (C1, C2 and C3) were used. Detached cultured HU-VEC-C cells were used as positive controls.

Figure 8b demonstrates lack of hPCLP antigens in mature peripheral blood leukocytes, as shown by FACS analysis (#015 = FITC-labelled anti-PCLP Ab, grey area). C1, C2 and C3 present FITC-labelled affinity purified rabbit antibodies against non-related antigens. The staining intensity of the peripheral blood leukocytes with #015 did not exceed that of controls.

Figure 9 shows a schematic drawing demonstrating the currently known expression of hPCLP in the hematopoietic cells of different species. In humans all CFU/BFU-staged cells, but not mature peripheral blood cells, are positive. The cells of earlier stem cell stages or the developing forms of the lymphoid lineage have not been studied yet. In thechicken, hPCLP is present in the early myeloid progenitor cells, but at the CFU/BFU-level hPCLP expression is restricted to the erythroid/megakaryoid lineages. Of the mature cells, only thrombocytes express hPCLP indicating that hPCLP is not a suitable stem cell marker. In the mouse, the embryonic hemangioblasts are hPCLP-positive, but neither further developmental stages nor the adult mice have been studied. In the rat, podocalyxin is expressed in the megakaryocytes and thrombocytes, but not in other peripheral blood leukocytes indicating that hPCLP is not a suitable stem cell marker in murine animals. Endothelial cells are hPCLP/podocalyxin-positive in all species.

Figure 10 shows the binding of deposited antibody ("BB8") and the 014 antibody to CFU/BFU lysates and to k562.

Figure 11 shows staining of k562 and BFU-E with BB8. Fig. 12 shows the reaction of monoclonal anti-k562 antibodies with the extracellular domain of human PCLP by immunoblotting.

The Detailed Description of the Invention

Definitions

In the present invention the terms used have the meaning they generally have in the fields of biochemistry, molecular biology, immunology, hematology, developmental stem cell identification, transplantation or grafting, recombinant DNA technology, includ-

ing related sciences, but some terms are used with a somewhat deviating or broader meaning than in the normal context. Accordingly, in order to avoid uncertainty caused by terms with unclear meaning some of the terms used in this specification and in the claims are defined in more detail below.

The term "hematopoiesis" means blood formation and includes the development and origin of blood cells including erythrocytes and all leukocytes such as granulocytes (neutrophiles, basophils, eosinophils), monocytes, lymphocytes (T cells, B cells, NK cells, dendritic cells), and megakaryocytes and platelets. Hematopoiesis takes place in two distinct steps, including primitive hematopoiesis and definitive hematopoiesis. The cells of the primitive hematopoiesis so called pluripotent stem cells are characterized by colony formation and are particularly useful as hematopoietic stem cells for transplants.

The term "hematopoietic tissues" means the tissues in which the hematopoiesis takes place. These tissues include in an adult person mainly bone marrow and lymphoid tissues, but in the fetus other areas including para-aortic tissues, aorta-gonad-mesonephric region, liver and placenta may participate in hematopoiesis.

The term "stem cells" or more particularly "hematopoietic stem cells" means self-renewing cells which have the capacity of forming more commited stem cells further giving rise to all different kinds of cells present in blood including both pluripotent stem cells and more or less committed progenitor cells. Stem cells in the present invention can be of different kinds. They can be totally undifferentiated, pluripotent stem cells examples of which include hemangioblasts, or partly differentiated committed progenitor cells including myeloid or lymphoid stem cells as well as colony forming CFU- and/or burst forming BFU cells. These colony- or burst-forming cells include a progenitor of granulocytes and monocytes (CFU-GM), a progenitor of platelets (CFU-MEG), a progenitor of granulocytes (CFU-G), a progenitor cell of monocytes (CFU-M), a progenitor of CFU-E capable of developing into erythrocytes (BFU-E) and a progenitor cell of eosinophils (CFU-Eo). Said partly differentiated stem cells can be found at different levels of differentiation including committed stem cells.

Stem cells can be recognized or identified with so called stem cell markers or colony formation assays. Stem cells are also defined as pluri-, toti- or multipotent.

The term "pluripotent" means that the hematopoietic stem cells have the capacity of being stem cells capable of developing into all different kinds of blood cells, including lymphocytes, monocytes, granulocytes, erythrocytes, megakaryocytes and platelets.

The term "multipotent" stem cells have the capacity to differentiate to more than one lineage, but they are already committed to certain lineages (for example myeloic stem cells or lymphoid stem cells).

The term "committed progenitor cells" means that the cells have lost the capacity of developing to certain blood cell types. In other words they are committed to certain lineages.

The term "mature blood cells" means blood cells which are fully differentiated and usually circulate in the peripheral blood or tissues. These cells are fully commited to their own lineage and cannot differentiate to cells of other lineages. Cell surface markers, which can be found also on mature blood cells are therefore not applicable as stem and/or progenitor cell markers.

The term "hematopoietic stem cell markers" means molecules, which are expressed by the pluripotent i.e. totally undifferentiated or more committed, partially differentiated hematopoietic stem cells, but not on differentiated mature blood cells. Particularly important are cell surface markers that can be identified by using binding substances, including antibodies and/or ligands which specifically recognize the stem cell marker, i.e. the cell surface antigen in question. None of the presently known marker molecules can alone be used for direct identification of pluripotent stem cells. At present the pluripotency has to be confirmed with CFU/BFU-assays which determines the colony-formation capacity. At present, the CD34 protein is the most commonly used hematopoietic stem cell marker for clinical purposes, although only a small percentage of hematopoietic CD34+ cells are true stem cells.

The term "CFU/BFU colony forming" means the colony-forming capacity, which is a characteristic feature of myeloic stem cells and can be assessed by the colony-forming assay (Ema et al., Blood, 75:1941-1946, 1990). The term "colony forming assay" means a method for confirming that potential stem cells identified with specific markers, really have the capacity of forming undifferentiated stem cells useful for transplants. In other words a confirmation assay of the value of preidentified stem cells.

The term "blood product" means bone marrow products, peripheral blood or placental blood and/or a preidentified stem cell population or individual stem cells. The term "mobilized blood cells" means blood cells, especially CD34 positive cells including pluripotent hematopoietic stem cells, which appear in the peripheral blood of the patients treated with hematopoietic growth factors.

The term "non-permeabilized cells" means cells with intact plasma membranes, i.e. plasma membranes that do not allow the entering of molecules like antibodies into the cell. "Permeabilized cells" mean cells that have been treated in a way which makes their plasma membranes "leaky". In other words, big molecules like antibodies can enter the cells.

The term "depleted cells" means cells which can be identified with certain stem cell markers, but lack the capacity of stem cell formation because they are partly differentiated. These depleted cells include partly differentiated cells but lack the marker which recognize a pluripotent stems cells. Even if such depleted cells cannot be used as transplants, they are however, useful as tools when studying blood cell differentiation and development (hematopoiesis) and can be used for future purposes in which the presence of pluripotent stem cells is unwanted. Such a situation might be e.g. a temporary need for white blood cell substitution in a patient with temporarily suppressed bone marrow. These depleted cells can be used as a reagent for providing test kits useful for studying the differentiation process in progenitor cells and for immune suppression, etc.

In the present invention "human hematopoietic podocalyxin-like

protein (hhPCLP)" means the protein present in the hematopoietic tissues and cells. Said hhPCLP is characterized by having in its glycosylated form an apparent MW of approximately 100-110 kDa depending on the method of determination and the cell types studied. A further bond occurs at around 63 kDa. Thereby, the MW differs substantially from that of other human PCLPs, which in their glycosylated form are characterized by having an apparent MW of at least 140 kDa. Furthermore hhPCLP is characterized by distinct antigenic epitopes, which most probably are due to post-translational modifications and can be demonstrated by its glycosylation pattern. Said hhPCLP protein has now been shown to be a useful marker for hematopoietic stem cells.

The term "human podocalyxin-like protein (hPCLP)" means the protein which is expressed in human glomerular podocytes and endothelial cells (Kershaw et al. 1997). It is a heavily glycosylated transmembrane protein having a molecular weight of 140 kDa or 155/165 kDa depending upon the cell type and the method of determination. In the present studies the apparent MW for the glomerular and endothelial PCLP was 155/165 kDa using 8 % SDS-PAGE gels and high molecular weight protein standards (Bio-Rad). The differences are probably due to different gel systems and MW markers used in the different studies.

The term "PCLP" includes PCLPs of all kinds of species and tissues. PCLPs have been isolated, purified and characterized from different tissues of different species, including humans, rodents and chicken. The protein part of said PCLPs have almost identical intracellular and transmembrane domains. The extracellular domains show less sequence homology, but are structurally similar having conserved cysteines in the membrane proximal part, and a serine-, threonine- and proline-rich domain typical for mucin-type glycoproteins. The hexose content of rat PCLP has been studied but nothing is known of the structures of the glycosyl groups of human PCLP. Since only one PCLP gene has been found, results indicate that PCLPs of different origin undergo significant cell type specific posttranslational modifications, e.g. glycosylation, sulphation etc. The estimated size of glomerular and/or endothelial tissue derived human PCLP calculated from the mRNA sequence is 54 kDa. The size of PCLPs of other species are also

around 55 kDa, when calculated from the mRNA or the corresponding amino acid sequences. (McNagny,et al., Journal of Cell Biology, 138:1395-1407, 1997; Kershaw, et al., J Biochem, 270:29439-29446, 1995; Kershaw, et al., J Biol Chem, 272:15708-15714, 1997). In all species, however, the expressed proteins detectable by antibodies have shown to have a much higher apparent MW as determined by Western blotting. It is to be noted that PCLP and hPCLP as such are not the object of the present invention. However, PCLP or hPCLP are useful for producing or manufacturing binding substances, such as antibodies or ligands for recognizing hhPCLP present on hematopoietic stem cells. As such PCLP and/or hPCLP are incorporated into the specification of the present invention.

The term "binding substances" means substances having an affinity for the extracellular parts of hhPCLP and include antibodies, ligands and parts thereof. Such binding substances can be found among antibodies recognizing the extracellular domains of hPCLP. Naturally, binding substances recognizing intracellular or transmembraneous domains can be used for recognizing hhPCLP, but they cannot be used for isolating stem cells. Said binding substances, especially the antibodies are capable of specifically recognizing the extracellular domains of PCLP and thereby facilitate specific detection, purification, identification, isolation and/or separation of hhPCLP expressing cells, the ultimate goal being isolation of pluripotent hematopoietic stem cells. Preferably, the binding substances are monoclonal or polyclonal antibodies raised against the extracellular domains of the PCLP itself, but the binding substances can also be other ligands such as lectins or fragments thereof.

The term "binding substances" also includes specific nucleotide sequences, e.g. antisense cDNA sequences, which are capable of recognizing mRNA expressed by the stem cells by hybridization based techniques. These sequences are useful as probes or primers and are mainly used for excluding the presence of hhPCLP mRNA. They can be used for identifying or recognizing stem cells, but not for isolating stem cells. If the mRNA expression is determined from a single cell, the cell in question might, however, be used for providing a stem cell transplant single cell cultivation.

The term "antibody" includes both monoclonal and polyclonal antibodies. Different kinds of antibodies both monoclonal and or polyclonal have been raised and new ones can be prepared by per se known conventional methods against PCLPs obtained from different species and different tissues. They can also be raised against synthetic peptides or recombinant proteins as described below.

The term "glycosylation patterns" means differences in the sugar side chains of protein in eukaryotic cells. Said differences may reflect different antigenic epitopes, functions, etc. Differences in glycosylation patterns can be demonstrated using antibodies, which recognize certain epitopes present in the glycosylated or non-glycosylated forms of the protein. Changes in the glycosylated parts of the protein or antigen, in this case hhPCLP can be obtained by using enzymes specifically degrading or adding certain sugar chains or by chemical means.

The term "screening" means methods with which stem cells easily can be identified and isolated using appropriate specific binding substances, including antibodies, ligands and/or nucleotide sequences, which recognize the presence of the hhPCLP in question or its mRNA. The binding substances, including antibodies or ligands, which can be used as such in free form or attached to a solid support or matrix are characterized by having the capacity of specifically binding to and recognizing hhPCLP and thereby binding stem cells expressing hhPCLP when contacted with the appropriate hematopoietic tissue. If a solid support is used the cells bound to solid support can be appropriately purified by eluting and washing with suitable diluents, including isotonic solutions, buffers, etc.

The term "solid support" means microparticles, beads, latex particles, magnetic particles, threads, pegs, tags, sticks, microwells, affinity columns, plates, etc. which are provided with the binding substances, which are capable of adhering to the surface of the solid support as such or when conjugated to the surface by per se known chemical or physical means. The binding can for example be facilitated by using appropriate affinity labels. The

solid supports can also be used for cultivating stem cells useful for transplants. Cultivation of all kinds of stem cells is possible, even if it is difficult for all lymphoid cells, which require elaborate cultivating conditions. For true stem cells the culture conditions are not exactly known.

The term "affinity labels" means binding substances, including antibodies or ligands with a high affinity to another substance. In other words, the affinity label is prone to form strong bonds with another substance. Suitable affinity labels can be found among affinity pairs, such as biotin-avidin / biotin-streptavidin. Strong bonds are formed between affinity pairs enabling the affinity-pair to act as means for assisting the capturing of the desired substances, in this case the antibody specifically binding to the extracellular parts of hPCLP and thereby to the cell which carries the hPCLP marker. Other synthetic or non-synthetic "affinity pairs" than the biotin-avidin pair mentioned above can also be applied. Suitable "affinity pairs" can be found among receptors and ligands, antigens andantibodies as well as among fragments thereof. The preferred "affinity labels" of the present invention include biotin, histidine oligomers, haptens, glycans, etc., whereas the preferred counterparts of the "affinity tags" are avidin, streptavidin, metal chelates, antibodies, lectins, etc.

The term "label-carrying molecule or tracer" means a binding substance provided with a marker molecule which makes the presence of the binding substance or the stem cell marker visible or detectable, i.e. recordable directly or indirectly when contacted with other reagents. Tracers or labels can be made recordable by their electrochemical or magnetic properties, fluorescence, luminescence, their infrared absorption, radioactivity or by enzymatic activity.

The General Description of the Invention

The present invention is related to a novel human hematopoietic tissue derived podocalyxin-like protein (hhPCLP) useful as a marker for identifying, purifying, isolating and producing hematopoietic stem cells. Said isolated and/or purified stem cells

provided with the hhPCLP marker are useful for providing hematopoietic stem cells for treating severe blood diseases, deficiencies or damages in the blood forming tissues. The present invention is also related to the use of hPCLP, especially the extracellular parts of hPCLP and most particularly hhPCLP for manufacturing as described thereinafter binding substances capable of specifically recognizing said marker on intact hematopoietic stem cells, but above all for identifying and isolating pluripotent hematopoietic stem cells with binding substances including antibodies, ligands or parts thereof having an affinity for and thereby recognizing hhPCLP on the surface of the intact stem and/or progenitor cells.

The hhPCLP-marker can also be used for assessing the quality of a stem cell population preidentified with other stem cell markers. This is achieved by determining the amount of useful hematopoietic stem cells present in a preidentified stem cell population. The present invention also provides methods for differentiating, separating and subsequently recovering different types of cells for different purposes.

The present invention is based on preliminary observations made with antibodies raised against the intracellular carboxyterminal domain of human PCLP (hPCLP). These preliminary results demonstrated that PCLP is an alternative marker for identifying pluripotent hematopoietic stem cells in a more effective manner than previously and to distinguish partially differentiated cells from the pluripotent stem cells. These preliminaryobservations were verified and are disclosed in the present invention. Said studies demonstrated that hhPCLP, and hhPCLP mRNA are expressed in colony forming cells, such as CFU-G, CFU-M, CFU-GM, CFU-MEG and BFU-E colony derived cells, as well as in 15 % of the CD34 positive cells from mobilized peripheral blood and thereby the cells are positive for PCLP. It was further demonstrated that mature peripheral blood leukocytes do not express PCLP protein and that erythrocytes and thrombocytes do not express PCLP protein or mRNA. These results demonstrated that hhPCLP is a new potential marker for developing hematopoietic stem cells.

In the present invention the expression of hhPCLP was for the

first time demonstrated in developing human blood cells. Because none of the mature human blood cells including isolated lymphocytes, monocytes or granulocytes expressed detectable amounts of PCLP and because human thrombocytes and erythrocytes were negative also at the mRNA level, it was demonstrated beyond doubt that the expression pattern of PCLP in adult human differs from the expression patterns observed in chicken or rat, in which PCLP has previously been demonstrated in progenitor cells bur also in mature thrombocytes.

The present invention provides a method for identifying hematopoietic stem cells at CFU/BFU stage as well as about 15 % of the mobilized CD34-positive cells. Thus, PCLP binding substances can be used to select defined hematopoietic stem cells from a CD34 positive stem cell population and for identifying, and isolating about 15 % pluripotent hematopoietic stem cells with CFU/BFU colony formation capacity present in a population of CD34 positive stem cells.

The identification can be carried out using per se known cell separation methods, preferably including the FACS method disclosed in Example 4. In the methods binding substances specifically recognizing hhPCLP, preferably antibodies raised against the extracellular domain of PCLP or hhPCLP should be used.

The preliminary results indicated that the PCLP-positive subpopulation may represent the colony forming cells, which can be identified among the CD34 positive stem cell population, since it has been shown beyond doubt that CFU-G, CFU-M, CFU-GM, CFU-MEG and BFU-E colony derived cells are positive for PCLP. Said results have been obtained with different presently available antibodies, but the results will be verified using antibodies raised against the extracellular domain of hhPCLP.

As stated above human hematopoiesis is still poorly understood, and more markers for cells at different developmental stages are needed. The present invention provides a new tool for studying hematopoiesis, because it is shown that human PCLP (hPCLP) is expressed in committed hematopoietic progenitor cells, but not in mature peripheral blood cells. Accordingly, it has been proved

that PCLP is a new valuable marker for this cell type and probably also for pluripotent stem cells. Simultaneously, with the separation of the hematopoietic stem cells, those cells which are not hhPCLP positive, i.e. depleted cells can be recovered and used for future uses such as for studying the progress of hematopoiesis i.e. partially differentiated stem cells at different stages of development.

Accordingly, the present invention provides an alternative method for isolating hematopoietic stem cells useful as transplants for treating blood diseases. In the method hematopoietic stem cells are identified by contacting a blood product, including mobilized or non-mobilized peripheral blood, placental blood or preidentified stem cell populations with a binding substance capable of specifically binding to and recognizing hhPCLP. The binding substances are preferably mono- or polyclonal antibodies, obtainable by per se known methods. Preferably the antibody is raised against an extracellular domain of hhPCLP. Specific ligands, such as lectins can also be used especially if the provision that they are not ligands having selectin activity is fulfilled. For identification of stem cells antisense cDNA probes hybridizing to mRNA can also be used.

The preliminary results were obtained using anti-PCLP antibodies of different specificities. It was demonstrated that the PCLP proteins of human hematopoietic stem cells, renal glomeruli, and cultured endothelial cells, had cell type specific modifications affecting their antigenic epitopes and apparent molecular size. The results demonstrated using immunological and biochemical techniques that hhPCLP is expressed in CFU- and BFU-staged hematopoietic progenitor cells of erythroid, megakaryoid and myeloid cell lineages and in 15% of CD34 positive cells of mobilized peripheral blood. Only mRNA was detectable in mature peripheral leukocytes. Additionally, by using anti-PCLP antibodies with different specificities, it is shown that the hematopoietic PCLP differs both in the antigenic epitopes and molecular size from the previously described glomerular or endothelial PCLPs. By Western blotting an affinity purified polyclonal antibody and the monoclonal antibody 1B6 recognized strongly a protein with an apparent MW of 63 kDa in hematopoietic cell lysates as well as in

the lysates of K562 leukemia cell line. In hhPCLP positive cell lysates a 63 kDa form was observed when stained with the polyclonal antibodies. At present little is known about the structure and properties of hhPCLP, but it is evident that it differs from other human PCLPs in respect of its molecular weight and glycosylation patterns as demonstrated with available PCLP antibodies and as described below.

As starting material in the preliminary studies polyclonal antibodies prepared from rabbit antiserum raised against a peptide containing the 19 carboxy-terminal amino acids of hPCLP were used. Suitable antisera could also have been raised in other animals, e.g. guinea pigs, mice, goats, etc. The rabbit antiserum marked as #488 was used in the examples described below.

In the preliminary studies the affinity purified anti-peptide antibody (#014) was prepared from rabbit antiserum (#488) raised against the peptide containing the 19 carboxy-terminal amino acids of an intracellular domain of hPCLP (GenBank™ accession number U97519). Said affinity purified polyclonal anti-hPCLP antibody (#014) was used to produce a FITC-conjugated anti-PCLP antibody (#015), which was also used for the identification and quantitation of PCLP-positive cells in fixed and permeabilized cell suspensions. A polyclonal anti-rat podocalyxin (#365) was also used for identifying hhPCLP.

Said polyclonal anti-peptide antibody #014 recognized all hematopoietic CFU- and BFU-staged stem cells, 15% of CD34 positive cells, and K562 leukemia cells by immunofluorescence, FACS and/or Western blotting analysis. Said polyclonal antibody recognized the 155/165 kDa doublet in the endothelial and glomerular cells as well as the 63 kDa form in the endothelial cells and hhPCLP. Said carboxyterminal intracellular domain used for immunization also shares a 6-amino acid sequence with the intracellular tail of endoglycan, but the polyclonal antibody did not bind to endoglycan, as shown in the examples. Said polyclonal antibody which was used to demonstrating the presence of hhPCLP on hematopoietic stem cells in their lysates is, however, not useful for isolating intact stem cells and/or committed progenitor cells.

For comparative reasons, monoclonal antibodies raised against human glomerular PCLP were used in the preliminary studies. The clones IB6 and GC7 are monoclonal antibodies recognizing their antigenic epitopes are carbohydrates (see below and Table 2), which differ from those of hhPCLP as demonstrated below, especially in the discussion about glycosylation patterns. It was shown that they bind to nonpermeabilized cells, which indicated their reactivity with the extracellular domain of PCLP. In glomerular lysates all these antibodies detected a protein doublet previously described for glomerular PCLP (Fig. 3a).

Monoclonal antibody PHM5 (Kerjaschki et al., J. Clin Invest, 78:1142-1149, 1986) raised against human glomerular extracts was used as a positive control. Said monoclonal antibody recognized hPCLP of renal glomeruli and endothelial cells, but did not bind to hhPCLP.

The polyclonal antibody #014 and the mAbs IB6 and GC7 all recognized in the glomerular extracts the 155/165 kDa form. The reaction of the polyclonal antibody was weak. The monoclonal antibody GC7 did not react with the hematopoietic cell lysates. In CD34 positive cell lysate, no clear reaction with the 63/55 kDa doublet was seen when stained with the polyclonal antibody. None of the peripheral blood cell extracts were positive when stained with the polyclonal antibody antiserum #488 (data not shown). The binding of the antibodies used for staining of the different sized PCLP proteins in hematopoietic CFU- and BFU -colony derived stem cells, endothelial cells, such as HU-VEC-C-cells and human glomerulus, as well as their binding intensities, are demonstrated in Table 1.

Table 1. Proteins recognised by anti-PCLP antibodies by Western blotting.

Antibodies against	Hematopoietic stem cells					HU-VEC-C				Glomerulus			
	kDa					kDa				kDa			
	165	155	63	55	119	165	155	63	55	165	155	63	55
Intra-cellular peptide #488 and #014	-	-	+++	+++	-	+	+	+++	+++	+	+	-	-
Glomerular PCLP													
mAb GC7	-	-	-	-	-	+++	+++	-	-	+++	+++	-	-
mAb 1B6	-	-	+++	+++	++	-	-	-	-	+++	+++	-	-

The staining intensity is demonstrated by a scale from negative (-) to intensive (+++).

By immunofluorescence, cells derived from the cultured CFU-G, CFU-M, CFU-GM, CFU-MEG and BFU-E colonies were positive for PCLP when cytocentrifuge slides were stained with #014 antibodies. K562 leukemia cells were positive (Fig. 2) as well and CD34 positive cells were positive (Fig. 2g). The staining intensity and the percentage of positive cells varied. The monoclonal antibody 1B6 stained the cells derived from the BFU-E colonies, but the staining obtained with the other CFU cells did not clearly differ from the non-specific background staining. The monoclonal antibody GC7 did not stain any of the stem cells. All cytocentrifuge slides of mature peripheral blood cells were negative with all tested anti-PCLP antibodies. These results obtained by immunofluorescence are demonstrated in Figure 2.

By FACS analysis the leukocyte populations of human peripheral blood were negative for PCLP when stained with the conjugated anti-peptide antibody (#015). However, the cells of the K562 leukemia cell line were positive. Of the mobilized peripheral blood CD34 positive cells, about 15% were positive for PCLP (Fig. 5).

The three categories of anti-PCLP antibodies (anti-peptide antibodies, and the two monoclonal anti-hPCLP antibodies) used in the present invention showed surprisingly different reactions with endothelial and hematopoietic PCLPs. None of the anti-PCLP antibodies could detect the 155/165 kDa PCLP doublet in the hematopoietic progenitor cells indicating that the hhPCLP differs structurally from other human PCLPs, probably due to its glycosylation pattern.

Glycosylation has been offered as one explanation for the discrepancy in molecular size and properties of hPCLPs. Human PCLP, for example, has five possible N-glycosylation sites and the mucine type domain has multiple potential O-glycosylation sites, and additionally it has one putative glycosaminoglycan attachment site (Kershaw, et al., J Biol Chem. 272:15708-15714, 1997). Evidence indicating that PCLP is heavily glycosylated exists, even though the sugar side chains of human PCLPs have not been analyzed. In the present invention some results obtained with different antibodies recognizing different epitopes and thereby capable of distinguishing between the glycosylation patterns are provided.

The glycosylation pattern of PCLPs seems to be very important when preparing different types of PCLP binding substances. For example, the extracellular mucin rich glycosylated part of PCLP seems to be the immunodominant area, as most antibodies made against whole, isolated PCLPs are species specific (Kershaw, et al., J Biol Chem, 270:29439-29446, 1995; Kerjaschki, et al., J Clin Invest, 78:1142-1149, 1986) hPCLP has not only species specific modifications, but also tissue specific modifications and affect the antigenic epitopes and probably the functions of human PCLP and hhPCLP.

The glycosylation patterns of different PCLPs can also be charac-

terized by the aid of antibodies and their capacity of binding or not binding to PCLPs after degradation of the glycosylated domains. For example, after pretreatment of the glomerular lysates with O-sialoglycoprotein endopeptidase (OSGE), the polyclonal anti-peptide antibody #014 gave a strong reaction with a PCLP degradation product of about 70 kDa, while the monoclonal antibodies IB6 and GC7 (Fig. 3a) did not detect this.

Also, the O-sialoglycoprotein endopeptidase treatment destroyed the 155/165 kDa doublet recognized by the monoclonal antibody GC7 in the endothelial cell lysate and diminished the 155/165 kDa doublet of the glomerular cell lysate, indicating that the monoclonal antibody GC7 recognizes epitopes in the sialomucin domain of PCLP.

The tissue binding activity of the monoclonal antibody 1B6 was lost when sialic acid was removed from the tissue by neuraminidase treatment removing sialic acid (Table 2). Also the monoclonal antibody IB6 did nor recognize the 70 kDa degradation product of glomerular PCLP obtained after O-sialoglycoprotein endopeptidase treatment.

As expected the affinity purified polyclonal antibody #014 is not affected by neuraminidase or metaperiodate treatments affecting the terminal parts of the glycosyl side chains (both N- and O-glycosylated). #014 is raised against a peptide made of the carboxyterminal 18 amino acids of the intracellular domain of hPCLP recognizing all forms of hPCLP having an intact COOH-terminus. It is characterized by reactivity degradation products of PCLP obtained with O-sialoglycoprotein endopetidase treated PCLP, which degrades mucin-like glycoproteins. In other words #014 does not recognize the hhPCLP, present in the cell surface.

The antigenic epitope of the monoclonal antibody GC7 is not sensitive for neuraminidase treatment but is partially sensitive for metaperiodate treatment, indicating that also its antigen is a sugar epitope. Furthermore when glomerular or HU-VEC-C-lysates were treated with O-sialoglycoprotein endopeptidase that only degrades mucin-type glycoproteins several degradation products were produced. Small products were formed from the original glomerular

hPCLPs above 140 kDa. All antibodies recognized these by Western blotting. In addition a new degradation product of approximately 70 kDa appeared.

Table 2. The effects of neuraminidase and metaperiodate treatment on the antigenic epitopes of frozen kidney cortex sections as by IF.

Abs	Untreated	Neuraminidase	Metaperiodate
1B6	+++	-	-
GC7	+++	+++	+
#014	+++	+++	+++

The staining is demonstrated by a scale from negative (-) to intensive (+++)

The preferred polyclonal or monoclonal antibodies used to recognize hematopoietic tissue derived stem cells and/or hhPCLP should preferably be raised against the extracellular part of the hhPCLP present on the surface of stem cells. The preferred antibodies of the present invention should not cross-react with the antibodies recognizing glomerular or endothelial hPCLP. However, antibodies cross-reacting with glomerular or endothelial PCLP can be used for recognizing hhPCLP as human blood does not contain any other cells than hematopoietic tissue derived stem cells, which have the hhPCLP marker. Therefore, any antibodies recognizing extracellular domains of hPCLP can be used for isolating, identifying and purifying hematopoietic stem cells from hematologic sources containing such cells.

Different kinds of antibodies, which can be used for demonstrating the hhPCLP-marker on cell surfaces have been produced and are available from different sources. Others with corresponding properties or totally new ones can readily be prepared using commonly used techniques. The antibodies are preferably raised against hhPCLP, but they can at least for research and verification purposes be raised even against intracellular or transmembranic domains of PCLP obtainable from different species and different

tissues. Suitable antibodies can be raised against synthetic peptides or recombinant proteins. New antibodies, both monoclonal and/or polyclonal can be raised against different PCLPs, having specific glycosylation patterns, such as the hhPCLP.

Polyclonal antibodies have been prepared and new ones can be prepared by immunizing suitable animals, including guinea pigs, mice, sheep, but preferably rabbits with peptides from PCLP, most particularly hhPCLP and the extracellular parts thereof. For example lysine-branched peptides are advantageous as described earlier (Miettinen et al., Am J Pathol. 154: 813-822, 1999). The rabbit antisera obtained is advantageously tested for PCLP-reactivity by peptide-ELISA-tests, by immunofluorescence at cryostat sections ofhuman kidney, at cytocentrifuge slides of human hematopoietic cells, and at slides of cultured HU-VEC-C-cells. Antibodies demonstrating the desired reactivity are affinity purified using suitable affinity columns, a particularly useful example is made of linear hPCLP peptides bound to matrices such as Sepharose 4B (Miettinen et al., Am J Pathol. 154: 813-822, 1999). The properties and suitability of the affinity purified antibodies are further tested by Western blotting at glomerular lysates, endothelial lysates, and at lysates of various hematopoietic cells. The affinity purified antibodies should also be tested for binding to the hhPCLP present at the cell surface of living hematopoietic cells, including CD34 positive cells and especially such CD34 positive cells, which have the colony formation capacity. This is preferably carried out using FACS technique.

In order to provide monoclonal antibodies more or less modified conventional hybridoma techniques are used. Mice are generally immunized with suitable PCLP, particularly hhPCLP derived peptides or recombiant proteins prepared in foreign bacteria. Lysine-branched peptides are particularly useful for obtaining monoclonal antibodies capable of recognizing the extracellular domains of hhPCLP. The mouse sera and the monoclonal antibodies are tested first for their reactivity with the immunizing peptides by using linear biotinylated peptides bound to streptavidin coated ELISA-plates as antigens. Monoclonal antibodies reacting with the peptides are further tested as described for the rabbit antibodies (see above).

cDNA clones of the entire extracellular domain of human PCLP fused to the the cDNA of glutathione transferase (GST) are transfected into suitable host specific expression vectors. The host cells can be mammalian cell lines, insect cells, fungal cells or bacterial cells, e.g. E. coli. The expression vector (pGEX-6P-2) is prepared for the expression of the fusion protein in E. coli DH5alpha-cells. Said bacteria are transformed with the expression plasmid, grown in suitable media, lysed and the fusion protein is isolated using standard molecular biology techniques. The purified fusion protein is isolated using suitable affinity columns, e.g. a glutathione column. GST is enzymatically separated from the fusion protein and the isolated extracellular domain is eventually used for immunization of mice to get more monoclonal antibodies capable of recognizing the extracellular part of hhPCLP.

Antibodies binding to the extracellular domain of living hematopoietic cells are used for isolation of hhPCLP positive cells from the CD34 positive cells obtained from mobilized peripheral blood, from placental blood, from bone marrow cells or from any source likely to contain hematopoietic stem cells. The cells are isolated based on the binding of the specific antibodies on their surface. The antibodies are first coupled with fluorescent dyes or with magnetic beads and correspondingly the cells to which the antibodies have bound areseparated by using cell sorting FACS machines or columns meant for isolation of magnetic bead-cells or by any suitable technique. The separated cells are further grown in vitro under circumstances in which they can differentiate to megakaryocytic, erythroid, myeloid or lymphatic lineages. By this stem cell capability of the hhPCLP-positive cells is proven.

The antibodies recognizing the extracellular domains of hPCLP are not specific for hhPCLP and therefore they will be used for the isolation of the mature, post-translationally fully modified hhPCLP from human hematopoietic cells. The isolated material can be used as antigen for the production of mono- and/or polyclonal antibodies recognizing only the antigenic epitopes specific for hhPCLP and not recognizing the PCLPs of other tissues such as kidney or endothelia. The immunization and characterization of the antibodies will be done as described below.

The isolated hhPCLP will be used also for experiments in which the specific antigenic epitopes are further characterized. These tests include analysis of the hexose content of the glycoprotein, making of glycopeptides and characterization of the sugar side chains etc.

Test animals can be immunized and boosted twice with the extracellular domain of hhPCLP obtainable from hematopoietic stem cell derived membranes. Affinity purified anti-hhPCLP antibodies can be obtained from the serum by using a suitable affinity column into which the immunisation peptide is coupled. The affinity purified antibodies can be neutralized with suitable buffers and the specificity of the anti-peptide serum antibodies can be used to characterize the antibody, e.g. by testing it on frozen sections of human kidney by immunofluorescence and Western blot analysis of human glomerular and endothelial cell lysates as well as hhPCLP. Some of the antibodies can be labeled with recordable labels, preferably fluorescent labels.

The clones giving identical binding pattern with the PHM5 antibodies (Kerjaschki et al., J. Clin Invest, 78:1142-1149, 1986) will be selected. Monoclonal antibodies, which have been characterized as being capable of specifically recognizing hhPCLP and not to cross-react with other human PCLPs will be used in the methods for identification, purification, isolation, differentiation and separation of the present invention.

For immunoprecipitation, human hematopoietic tissue lysates will be diluted in buffer and precleared by incubation with normal mouse serum. The precleared lysate will be further incubated overnight at low temperature with a suitable monoclonal antibody. The lysates will be incubated with anti-mouse IgG-agarose and the bound immune complexes are then collected by centrifugation. The washed agarose beads are suspended under reducing conditions, boiled and analysed e.g. by PAGE and Western blotting.

For identification and characterization immunofluorescence staining experiments, cytocentrifuge slides can be prepared from freshly isolated hematopoietic stem cells, identified as such by

the colony-formation assay. The cells can be grown on glass coverslips. The slides and coverslips are fixed and stained with appropriate primary antibodies including monoclonal antibodies for characterization as well as with suitable polyclonal anti-rat podocalyxin and secondary antibodies including conjugated IgGs.

hhPCLP positive stem cells can be further characterized by confocal microscopy and stained as living cells on coverslips by keeping them on ice during the procedure and by using cold reagents. The staining protocol is the same as for the prefixed samples. The cells are fixed after staining.

For the cell purification experiments, e.g. with FACS, whole peripheral blood samples can be stained with e.g. anti-CD45 coupled with peridin chlorophyll protein with either phycoerythrin (PE) coupled anti-CD3 or anti-CD20. The cells should preferably be washed and fixed for the staining experiments using anti-extracellular hhPCLP antibodies, the cells can advantageously be permeabilized. Thereafter the cells can be stained with a conjugated anti-hhPCLP antibody or with affinity purified control conjugates. The cells can be incubated, washed and thereafter preserved in a suitable medium until analyzed using a flow cytometer. For the hhPCLP positive cells the staining procedure is substantially identical with the methods used for the identifying and characterizing peripheral blood leukocytes. Accordingly, cells are fixed and permeabilized, but only conjugated anti-hhPCLP antibody and different controls for CD34 positive and K562 cells will be used.

For characterization with SDS-PAGE, the isolated hhPCLP positive cell populations will be lysed using sodium deoxycholate and suitable buffers, supplemented with a cocktail of enzyme inhibitors. The cells will be suspended in suitable solution, boiled and run under reducing conditions using gels and a protein Minigel electrophoresis system.

In order to obtain membrane fraction lysates, the hematopoietic stem cells are suspended in the homogenisation buffer including sugar, homogenized by hand in a glass homogenizer and centrifuged. The supernatant is further centrifuged and the pellet is

suspended.

For Western blotting, the separated hhPCLPs are transferred to nitrocellulose membranes using an appropriate blotting apparatus. The nitrocellulose strips are reacted with the selected specific monoclonal antibodies. They are washed and incubated with label provided tracers, such as horseradish peroxide-coupled anti-mouse or anti-rabbit IgG, and the bound antibodies are visualized using suitable techniques, for example enhanced chemiluminescence technique when using horseradish peroxidase.

Using immunofluorescence on hematopoietic stem cells identified by the colony formation assay, the anti-hhPCLP extracellular peptide antiserum and the affinity purified anti-peptide antibodies as well as the monoclonal anti-hhPCLP IgGs should stain the hematopoietic stem cells in a manner typical for other known anti-podocalyxin antibodies. By Western blotting on glomerular extracts, all antibodies should recognize the hhPCLP protein having an apparent MW of 100-110 kDa.

To demonstrate that the rabbit anti-intracellular peptide antibodies and the monoclonal IgGs recognize the same protein, human hematopoietic stem cell lysate can be immunoprecipitated with monoclonal antibody.

An alternative method for recognizing or identifying hhPCLP was provided by the use of nucleotide sequences and hybridization methods and/or PCR-techniques. Useful probes capable of identifying stem cells expressing hhPCLP were obtained from the corresponding mRNA by RT-PCR (reverse transcription-PCR). For this purpose mRNA was purified from the hematopoietic stem cells by using per se known mRNA isolation and purification systems, e.g. commercially available mRNA isolation kits. The isolated mRNA was treated with DNAse and RT-PCR was performed using random hexanucleotide primers and reverse transcriptase.

Specific primers were designed for hhPCLP using the known sequence deposited at GenBank™ (accession number U97519). The primers include different sense and antisense primers in the first round of PCR and nested sense and antisense primers in the second

round of PCR. The specificity of the primers to PCLP was confirmed by using the protein database BLAST search. Primers for beta-actin were used as controls.

The cDNAs were amplified in the presence of the selected primers, polymerases and other appropriate reagents using PCR systems well known in the art. The PCR product was analyzed by per se known methods including agarose gel electrophoresis and ethidium bromide staining, purified and sequenced. Sufficiently long, about 15-20 nucleotides long probes comprising said antisense sequences can be used for identifying hhPCLP mRNA.

The RNAs extracted from the cells of the cultured CFU-G, CFU-M, CFU-GM and BFU-E colonies as well as from the cells of CFU-MEG-cultures were positive for hhPCLP mRNA as shown by nested RT-PCR (Fig. 4a). Of the isolated peripheral blood cells, erythrocytes and thrombocytes were negative, but CD19 positive cells (B-lymphocytes), CD3 positive cells (T-lymphocytes), CD14 positive cells (monocytes) and granulocytes were positive. Similarly, the CD34 positive cells of mobilized peripheral blood were positive. Also the RNA isolated from the K562 leukemia cells contained PCLP mRNA (Fig 4b). All the RT-PCR-controls were negative (data not shown).

In conclusion the present invention is related to the use of PCLP, particularly hhPCLP, as a marker for identifying, isolating and manufacturing hematopoietic progenitor cells useful as transplants for treating conditions with depressed function of the blood forming tissues. The preliminary identification was performed primarily using antibodies raised against an intracellular domain of hPCLP, but will be verified with antibodies raised against living pluripotent hhPCLP expressing cells.

The preliminary studies are described in more detail in the experimental part below.

E x a m p l e s

Example 1
Cells and tissues

The cells from CFU-GM, CFU-G, CFU-M and BFU-E colonies were obtained from in vitro cultures of normal human bone marrow mononuclear cells (MNC) cultured for routine purposes as previously described (Juvonen, et al., Blood, 78:3066-3069, 1991). Briefly, 1-2 ml of normal bone marrow obtained from healthy donors was diluted in Iscove's modified Dulbecco's medium (IMDM, Life Technologies Ltd., Paisley, UK) containing preservative-free heparin per 100 U/ml. The semisolid culture medium for granulocyte-macrophage and erythroid progenitors consisted of 20% of fetal calf serum (HyClone, Logan, Utah, USA), 1% delipidated and deionized bovine serum albumin, 10^{-4} M mercaptoethanol (Fluka, Buchs, Switzerland), 310 µg/ml fully iron-saturated human transferrin (Sigma Chemical Company, St Louis, Missouri, USA), 20% human phytohemagglutinin-stimulated leukocyte-conditioned medium, and 0.8% methyl cellulose (Methocel, Fluka) in IMDM. The growth of erythroid colonies was stimulated with 2 U/ml of recombinant human erythropoietin (Eprex^R, Janssen-Cilag) and the growth of granulocyte/macrophage colonies with bladder cancer cell line 5637-conditioned medium. The concentration of MNCs in the cultures was $5x10^4$/ml and the culture plates were incubated for 14 days at 37°C in a fully humidified atmosphere with 5% CO_2. The colonies for CFU-GM, CFU-G, and CFU-M were identified by inversion microscopy based on the typical morphology of the cells. The colonies of BFU-E were identified by their red color. Individual colony types (erythroid, pure granulocytes, pure macrophages, and mixed type of granulocyte-macrophage colonies) were picked up separately with a thin glass pipette under microscopic observation for further studies.

The CD34-positive (CD34+) cells were collected from mobilized peripheral blood MNCs obtained from autologous transplants from patients with multiple myelomas. The purification of the CD34+ cells was proceeded according to standard methods of CliniMACS (Miltenyi Biotech GmbH, Bergisch Gladbach, Germany) by using

anti-CD34 magnetic beads (mean 93.5% purity).

For the CFU-MEG (megakaryocytic) cultures, mobilised peripheral blood CD34+ cells (1 x 10^4 / ml) were cultured for 7 days in 6-well culture plates (CellStar, Greiner, Germany) in 5 ml of StemSpan SFEM medium (StemCell Technologies, Vancouver, Canada) supplemented with 10 ng/ml of human IL-3 and IL-6 and 50 ng/ml of thrombopoietin (StemCell Technologies). At day 7, CD41+ cells were isolated using FITC-conjugated anti-CD41 Abs (1µg/1x10^6 cells, Caltag Laboratories, Burlingame, CA) and anti-FITC magnetic beads with miniMACS purification columns (Miltenyi Biotech GmbH). A test sample of the cells was stained with phycoerythrine (PE)-conjugated anti-CD34 Abs (Anti-HPCA-2, Becton Dickinson Immunocytometry Systems, San Jose, California, USA) on cytocentrifuge slides to ensure that the purified cells were of type CD41+CD34+.

The normal, peripheral blood cell populations were separated into MNCs and granulocytes by gradient centrifugation (Ficoll-paque, Pharmacia Biothec, Uppsala, Sweden). The MNCs were then isolated further by using specific FITC-conjugated Abs (2-4 µl/10^6 positive cells): anti-CD3 for T-lymphocytes, anti-CD19 for B-lymphocytes, anti-CD14 for monocytes, (Becton Dickinson Immunocytometry Systems) and anti-FITC magnetic beads with miniMACS purification columns (Miltenyi Biotech GmbH). The Ficoll -separated granulocytes were incubated with the same Abs, and the cells that did not attach to any of the used magnetic beads (the negative fraction) were collected. All samples were purified twice through the columns.

Cells from a chronic myelogenous leukemia cell line K-562 (ATCC CCL 243), that have been shown to be able to develop into granulocytic, monocytic, erythrocytic or megakaryocytic lineage depending on the stimulation were cultured in RPMI supplemented with 10% FBS, L-glutamine 0.3 mg/ml, penicillin 100 U/ml, and streptomycin 100 µg/ml. Cells of a human umbilical vein endothelial cell line (HU-VEC-C, ATCC CRL 1730), that were cultured in the same RPMI medium as the K562 cells were used as positive controls. The kidney tissue for immunofluorescence staining experiments and the isolation of glomeruli extracts was obtained from

the normal parts of removed kidneys of renal cancer patients, as described earlier (Holthofer, et al. Kidney Int, 45:123-130, 1994). The study was approved by the ethical committee of Helsinki University Central Hospital.

Example 2
Nested RT-PCR

The RNA for the reverse transcriptase-PCR (RT-PCR) samples was purified from the hematopoietic cells by using a µMACS mRNA isolation kit (Miltenyi Biotech GmbH) and from all other samples by using TRIzol (Life Technologies, Gibco BRL Inc, Gaithersburg, New Jersey, USA). The isolated RNA was treated with DNAse (RQ1, Promega, Madison, Wisconsin, USA) and reverse transcribed using random hexanucleotide primers (Boehringer Mannheim, Germany) and MMLV reverse transsscriptase (Promega). For the RT-negative controls (RT-), no MMLV was added.

The following primers were designed for human PCLP: For the first round, sense primer 5' -CCG TGG TCG TCA AAG AAA TC (SEQ ID NO:3:) corresponding to nucleotides 1380 to 1399, and antisense primer 5'GTC GTC CTT GGT CAG GTT GT (SEQ ID NO:4:) corresponding to nucleotides 1788 to 1807. For the second round, nested sense primer 5' -GAC ATG AAG CTA GGG GAC CA (SEQ ID NO:5:) corresponding to nucleotides 1484 to 1503, and nested antisense primer 5'TTG AGG CTG ACC ACC TTC TT (SEQ ID NO:6:) corresponding to nucleotides 1733 to 1752 (numbering according to GeneBankTM accession number U97519) (Fig.1). The specificity of the primers to PCLP was confirmed by using the protein database BLAST search. The beta-actin gene was used as control.

The cDNAs were amplified (total volume 2µl) in the presence of 0,5 M primers, 0,2 mM dNTP (Finnzymes, Espoo, Finland), 10X PCR buffer with 15 mM $MgCl_2$ (Perkin-Elmer, Cetus, Conneticut, USA) and 0,6 U AmpliTaq polymerase (Perkin-Elmer). The following program was used: first denaturation at 95°C for 5 minutes followed by 30 cycles: denaturation for 30s at 94°C, annealing for 30s at 55°C for 1st round, 57°C for nested primers, and extension at 72°C for 30s, and then finally 72°C for 10 minutes. The PCR product was analyzed by agarose gel electrophoresis and ethidium bro-

mide staining, purified (QIAquik Gel Extraction Kit, Qiagen, Germany) and sequenced directly using gene-specific primers (ABI-Prism 310, Perkin Elmer applied Biosystems, California, USA). Due to the small amount of cells available (1-5 x 10^4 cultured stem cells/sample), the RNA levels were not measured.

Example 3
Anti-human PCLP Abs

Two rabbits were immunized with multiple subcutaneous injections of 1 mg (in 0,5 ml PBS) of lysine-branched 19 amino acid peptide (SEQ ID NO :1:) having the amino acid sequence VPLDNLTKDDLDEEEDTHL, corresponding to the intracellular carboxy terminus of human podocalyxin (Fig. 1), and mixed with 0.5 ml Freund's complete adjuvant (Difco Laboratories, Detroit, Michigan, USA). The rabbits were boosted twice with 1 mg of the same peptide, mixed with 0,5 ml of Freund's incomplete adjuvant, at 4 and 7 weeks after the initial immunisation. The first serum samples (#487 and #488) were collected at one week after the 1st boost and the final collections were performed 2 weeks from the 2nd boost. An affinity purified anti-human PCLP Ab (#014) was obtained from the serum (final collection #494) by using a CNBr-activated sepharose 4B affinity column (Amersham Pharmacia Biotech, Uppsala, Sweden) into which 2 mg of the linear form of the immunisation peptide was coupled (total volume 2 ml). The affinity purified monospecific Ab was eluted with 0.1 M glycine-HCl, pH 2.7, neutralized immediately with 1.5 M Tris-HCL pH 8, and concentrated to 0.38 mg of IgG/ml. The specificity of the anti-peptide serum Abs #488 and affinity purified #014 was tested on frozen sections of human kidney by immunofluorescence (IF) and Western blot analysis of human glomerular and HU-VEC-C cell lysates. Some of #014 Abs were labeled with FITC (Sigma Chemical Company) as described earlier. The FITC/protein ratio (Abs495/Abs280) of the conjugated antibodies (#015) was 2.5.

Monoclonal anti-human PCLP antibodies were made according to the protocol described for anti-rat podocalyxin Abs. The clones giving identical binding pattern with the PHM5 (Kerjaschki et al., J. Clin Invest, 78:1142-1149, 1986) antibodies (Kerjaschki et al., J. Clin Invest, 78:1142-1149, 1986) were selected. Mono-

clonal Abs GC7 IgG and IB6 IgG were used in this study. For specificity of these antibodies see Example 6.

For immunoprecipitation, human glomerular and HU-VEC-C cell RIPA-lysates (see Western blotting analyses) were diluted 1/5 TBS (50 mM Tris-HCL, 150 mM NaCl, pH 8) and precleared by incubation with normal mouse serum (50 µl serum/1 ml lysate). The precleared lysate was further incubated overnight at +4°C with mAb GC7 (1 µg/ml). The lysates were incubated with anti-mouse IgG-agarose (Sigma Immuno Chemicals) on ice for 1 h, and the bound immune complexes were collected by centrifugation. The washed agarose beads were suspended in 4x reducing Laemmli sample buffer (RLSB), boiled for 5 min and analysed by PAGE and Western blotting.

Example 4
Immunofluorescence and FACSR

For immunofluorescence staining experiments, cytocentrifuge slides were prepared from freshly isolated cells suspended in IMDM (the methylcellulose cultured stem cells) or in PBS (800g, 4 min, Cytospin, Shandon, Pensylvania, USA). HU-VEC-C cells were grown on glass coverslips. The slides and coverslips were fixed in acetone at -20°C or in 3% paraformaldehyde (PF) in PBS for 10 min and stained with the primary (#014, mAbs IB6 and GC7 and polyclonal anti-rat podocalyxin #365 in PBS-3% BSA) and secondary Abs (FITC-conjugated rabbit anti-mouse IgG, 50 (F0261, DAKO A/S, Glostrup, Denmark; FITC-conjugated goat anti-mouse IgG, Jackson ImmunoReseacrh Laboratories, West Grove, Pensylvania, and swine anti-rabbit IgG conjugated with TRITC, R0156, DAKO), as described (Miettinen, et al., Am J Pathol, 154:813-822, 1999; Miettinen, et al., Am J Pathol, 137:929-944, 1990). For the confocal microscopy experiments, HU-VEC-C cells were stained as living cells on coverslips by keeping them on ice during the procedure and by using cold reagents. The staining protocol was the same as for the prefixed samples. The cells were fixed with 3% PF after staining.

For the FACSR experiments, whole peripheral blood samples were stained at 4°C for 30 min with anti-CD45 coupled with peridin chlorophyll protein (Per CP), with either PE-coupled anti-CD3 or

-CD20, 10 µl of each Ab/100 µl blood (Becton Dickinson). The cells were washed twice with PBS and fixed in 3% PF at room temperature for 15 min, and then washed twice with PBS. For staining experiments with the anti-intracellular peptide Abs the cells were further permeabilized in PBS into which 0,05% saponine and 5% normal rabbit serum was added at 4°C for 10 min. Then the cells corresponding to 100 µl of original blood were stained with 2,5 µg of FITC-conjugated anti-PCLP #015 or with affinity purified control conjugates including rabbit anti-chicken IgG, anti-mouse IgG (Jackson ImmunoResearch Laboratories) or rabbit anti-goat IgG (Cappel, Costa Mesa, California,USA). The cells were incubated at 4°C for 30 min, washed twice and then preserved in 1%PF-PBS until they were analyzed using Becton Dickinson FACScan flow cytometer For the CD34 positive and K562 cells, the staining procedure was similar. CD34 positive and K562 cells were stained with the same antibodies as all other cells: i.e. with #015 and with the control FITC-conjugates.

Example 5
Western blotting analysis

For SDS-PAGE, the isolated cell populations or human glomeruli were lysed in RIPA -lysis buffer (TBS, 1% NP-40, 0,5% Sodium deoxycholate, 0,1% SDS) supplemented with a cocktail of enzyme inhibitors (Complete™, Boehringer Mannheim, Germany), suspended in 4x reducing Lammli sample buffer (RLSB), boiled for 10 min, and then run under reducing conditions using 8% gels and a protein Mini-gel electrophoresis system (Bio-Rad Laboratories, California, USA). For the membrane fraction lysates, HU-VEC-C cells were suspended in the homogenisation buffer (TBS with 125 mM sucrose), homogenized by hand in a glass homogenizer, centrifuged at $600g_{max}$ at +4°C, for 10 min. The supernatant was further centrifuged at 100 $000g_{max}$ for 1h and the pellet was suspended in RIPA-buffer.

For Western blotting, the separated proteins were transferred to nitrocellulose sheets using a Novablot semidry blotting apparatus (Amersham Pharmacia Biotech). The nitrocellulose strips were reacted with the antibodies #488, #014, monoclonal antibodies 1B6 or mAb GC7, washed and incubated with HRP-coupled anti-mouse or anti-rabbit IgG (P0260 or P0217, DAKO), and the bound Abs were

visualized with the ECLTM technique (Amersham Life Science, Buckinghamshire, UK).

Example 6
Specificity of the anti-PCLP antibodies

By immunofluorescence on frozen sections of human kidney, the anti-human PCLP intracellular peptide antiserum (#488) and the affinity purified anti-peptide Abs (#014), as well as the monoclonal anti-glomerular PCLP IgGs (mAbs 1B6 and GC7), stained glomerular podocytes and renal endothelial cells in a manner typical for anti-podocalyxin Abs (Miettinen, et al., Am J Pathol, 154:813-822, 1999) (Fig. 2i and j). By Western blotting on glomerular extracts, all Abs recognized a doublet of proteins with an apparent MW of 165 and 155 kDa, as described earlier for anti-PCLP (Kerjaschki, et al., J Clin Invest, 78:1142-1149, 1986) (Fig. 3a).

To demonstrate that the rabbit anti-intracellular peptide Abs and the monoclonal IgGs recognize the same protein, human glomerular lysate was immunoprecipitated with monoclonal antibody GC7. By Western blotting mAb 1B6 and #014 recognized the 155/165 kDa doublet in the glomerular precipitate and #014 recognized the doublet also in HU-VEC-C precipitate (Fig. 4).

Cultured acetone fixed HU-VEC-C cells also stained with #014, mAb GC7 and also weakly with mAb 1B6 by immunofluorescence. When the cells were fixed with PF, which makes the cells impermeable so that only proteins outside the plasma membrane are stained, equal staining as compared with acetone fixed cells was obtained with mAb GC7 but #014 did not stain the cells (Fig. 2K and 2L). In order to obtain more specific evidence about the recognition sites of the Abs, HU-VEC-C cells were stained alive so that the cell membrane would stay intact and endocytosis of the Abs was prevented by keeping the cells on ice. The stained cells were studied under confocal microscope: mAb 1B6 and mAb GC7 both showed positive staining (although the staining of mAb 1B6 was weaker) around the plasma membrane. All stained with #014 which has an intracellular binding site were completely negative. The epitopes of the monoclonals are extracellular (data not shown).

By Western blotting, mAb GC7 recognized a high molecular weight doublet in HU-VEC-C cell lysates similar to the glomerular extracts (Fig. 3a and 3b). In contrast, the intracellular peptide Abs and mAb 1B6 recognized strongly a smaller band with an apparent MW of 63 kDa in HU-VEC-C cell lysates. The smaller band was also seen as a doublet of 63/55 kDa when stained with the anti-peptide Abs, but only the upper 63 kDa band was recognized by mAb 1B6 with no binding to the 155/165 kDa form, whereas #014 reacted weakly with the larger doublet (Fig.3A and 3B). When lysates of HU-VEC-C cell membrane fractions were immunostained with #014, the high molecular weight doublet was recognized more intensively (data not shown). When HU-VEC-C lysates were immunoprecipitated with mAb GC7 and then stained by Western blotting with #014 and mAb GC7 the 155/165 kDa doublet was recognized, whereas staining with mAb 1B6 resulted in no binding to the precipitated antigens.

The binding of #488 Abs to the 155/165 kDa doublet of glomerular lysates and to the 63/55 kDa doublet of HU-VEC-C cell lysates was specific, as preincubation of the Abs with the intracellular peptide used for the immunisation, but not with an unrelated peptide, inhibited binding (Fig. 3c).

Example 7

Human PCLP mRNA in hematopoietic cells

The RNAs extracted from the cells of the cultured CFU-G, CFU-M, CFU-GM and BFU-E colonies as well as from the cells of CFU-MEG-cultures were positive for PCLP mRNA as shown by nested RT-PCR (Fig. 4a). Of the isolated peripheral blood cells, erythrocytes and thrombocytes were negative, but CD19+ cells (B-lymphocytes), CD3 positive cells (T-lymphocytes), CD14+ cells (monocytes) and granulocytes were positive. Similarly, the CD34+ cells of mobilized peripheral blood were positive. Also the RNA isolated from the K562 cell line cells contained PCLP mRNA (Fig 4b). All RT-controls were negative (data not shown).

Example 8

PCLP antigens in hematopoietic stem cells

By immunofluorescence, cells derived from the cultured CFU-G, CFU-M, CFU-GM, CFU-MEG and BFU-E colonies were positive for PCLP when cytocentrifuge slides were stained with the affinity purified intracellular peptide Abs. K562 leukemia cell line cells were positive as well (Fig. 2). Also, CD34+ cells were positive, but the staining was more localized at the membrane as compared with the staining of all the other cells (Fig. 2g). The staining intensity and the percentage of positive cells varied. The staining intensity of the CFU-G cultures and the CD34+ cells was weakest (Fig. 2). Of the monoclonal antibodies 1B6 stained the cells derived from the BFU-E colonies whereas it did not give a clear reaction with the other cell types and mAb GC7 did not stain any of the stem cells (Fig. 2a- Fig. 2c). All cytocentrifuge slides of peripheral blood cells were negative with all tested anti-PCLP Abs.

By FACS analysis the leukocyte populations of human peripheral blood were negative for PCLP when stained with the FITC-conjugated anti-peptide Ab #015. However, the K562 cells were positive. Of the mobilized peripheral blood CD34+ cells, about 15% were positive for PCLP (Fig. 5).

By Western blotting, anti-intracellular peptide Abs (serum #488 and affinity purified #014) detected the 63/55 kDa doublet and however, mAb 1B6 only detected the 63 kDa protein in the extracts of CFU-GM, CFU-M, BFU-E and CFU-Meg culture derived cells (Fig. 6), while mAb GC7 did not react with the extracts. Additionally, mAb 1B6 bound to a 119 kDa double band in all stem cell extracts except the CFU-G extract (Fig. 6). In the K562 cellextracts, Ab #014 also recognized the 63 kd protein. In CD34+ cell lysate, no clear reaction with the 63/55 kDa doublet was seen when stained with Ab #014. None of the peripheral blood cell extracts were positive when stained with Ab #488 (data not shown). The binding of the Abs used to staining of the different sized PCLP proteins in hematopoietic CFU- and BFU -colony derived stem cells, HU-VEC-C cells and human glomerulus, as well as their binding intensities, are demonstrated in table 1.

Example 9
Synthetic 19 amino acid peptides

In order to get antibodies capable of binding to the extracellular part of hhPCLP on living cells synthetic 19 amino acid peptides of the membrane proximal to the non-glycosylated extracellular domain of PCLP for example (SEQ ID NO:2:) having the following sequence KLPAKDVYERLKDKWDELK are made based on the published human PCLP sequence data (GeneBankTM accession number U97519). The primers used for PCR were as follows:

sense primer 5' -CCG TGG TCG TCA AAG AAA TC corresponding to nucleotides 1380 to 1399 (SEQ ID NO:3:)
antisense primer 5' GTC GTC CTT GGT CAG GTT GT corresponding to nucleotides 1788 to 1807 (SEQ ID NO:4:)
nested sense primer 5' -GAC ATG AAG CTA GGG GAC CA corresponding to nucleotides 1484 to 1503 (SEQ ID NO:5:)
nested antisense primer 5' TTG AGG CTG ACC ACC TTC TT corresponding to nucleotides 1733 to 1752 (SEQ ID NO:6:)

In addition the whole extracellular part of hPCLP introduced plasmid will be transformed in E. coli and the protein will be used for antibody preparation.

Example 10
Rabbit antibodies

Rabbits were immunized with lysine-branched peptides, as described earlier (Miettinen et al. Am J Pathol. 154: 813-822, 1999). The rabbit antisera are tested for PCLP-reactivity by peptide-ELISA-tests by immunofluorescence at cryostat sections of human kidney, at cytocentrifuge slides of human hematopoietic cells, and at slides of cultured HU-VEC-C-cells. Specific antibodies are affinity purified using affinity columns made of the corresponding linear hPCLP peptides bound to matrices such as Sepharose 4B (Miettinen etal. Am J Pathol. 154: 813-822, 1999). The affinity purified antibodies are further tested by Western blotting at glomerular lysates, endothelial lysates, and at lysates of various hematopoietic cells, and tested for binding to the

hhPCLP present at the cell surface of living hematopoietic cells (such as CD34+ cells) by using FACS technique.

Example 11
Monoclonal antibodies

Mice were immunized with lysine-branched peptides to obtain monoclonal antibodies capable of recognizing the extracellular domains of hhPCLP. The mouse sera and the monoclonal antibodies are tested first for their reactivity with the immunizing peptides by using linear biotinylated peptides bound to streptavidin coated ELISA-plates as antigens. Monoclonal antibodies reacting with the peptides are further tested as described for the rabbit antibodies (see above).

Example 12
The use of fusion protein

A cDNA clone of the entire extracellular domain of hPCLP, combined with glutathione transferase (GST) in an expression vector (pGEX-6P-2) for the expression of the fusion protein in E. coli DH5alpha cells is done. The bacteria is transformed with the expression plasmid, grown in suitable media, lysed and the fusion protein is isolated using standard molecular biology techniques. The purified fusion protein is isolated using glutathione column, GST is enzymatically separated from the fusion protein and the isolated extracellular domain is eventually used for immunization of mice to get more monoclonal antibodies recognizing the extracellular part of hhPCLP, as described above.

Example 13
Testing of stem cell capability of the hhPCLP-positive cells

The antibodies binding to the extracellular domain of hhPCLP at hematopoietic cells are used for isolation of the hhPCLP-positive cells from the CD34-positive cells obtained from mobilized peripheral blood, from placental blood, from bone marrow cells, or from any source likely to contain hematopoietic stem cells. The cells are isolated based on the binding of the specific antibodies on their surface. The antibodies are first coupled with fluo-

rescent dyes or with magnetic beads and correspondingly the cells to which the antibodies have bound are separated by using cell sorting FACS machines or columns meant for isolation ofmagnetic bead-cells or by any suitable technique. The separated cells are further grown in vitro under circumstances in which they can differentiate to megakaryocytic, erythroid, myeloid or lymphatic lineages. By this stem cell capability of the hhPCLP-positive cells is proven.

Example 14
The characterization of the specific antigenic epitopes

As the antibodies recognizing the extracellular domains of hPCLP are not specific for hhPCLP they are used for the isolation of the mature, post-translationally fully modified hhPCLP from human hematopoietic cells. The isolated material is used as antigen for the production of monoclonal and/or polyclonal antibodies recognizing only the antigenic epitopes specific for hhPCLP and not recognizing the hPCLPs of other tissues such as kidney or endothelia. The isolated hhPCLP is used also for experiments in which the specific antigenic epitopes are further characterized. These tests include analysis of the hexose content of the glycoprotein, making of glycopeptides and characterization of the sugar side chains etc.

Example 15

New monoclonal antibodies were raised against K562 cells, which according to previous data express PCLP at their surface. The idea was to raise antibodies that recognize the glycosylated form of PCLP typical for leukocytes/hematopoietic stem cells. By this method 4 clones (CF9 B8, BE6 D9, BB8 BB11, CC9E9) were obtained that a) by an ELISA assay recognize extracellular peptides of hPCLP, b) by the indirect immunofluorescence test react with cytocentrifuge preparations of K562 cells, and c) by immunoblotting of cell extracts of CFU-/BFU-staged hematopoietic stem cells recognize proteins also recognized by affinity-purified #014 anti-PCLP antibodies (made against an intracellular PCLP peptide). Thus, these clones are anti-PCLP antibodies and also anti-K562 antibodies.

Using these MAbs and the magnetic bead techniqe (anti-mouse IgG and anti-mouse IgM beads; Miltenyi) cells binding to these MAbs were isolated from a peripheral blood mononuclear cell fraction. The PBMCs were from a patient treated for the mobilization of bone marrow CD34+ cells (also including some pluripotent hematopoietic stem cells) into the peripheral blood. The mobilization treatment of the patient was given in the Department of Hematology, Helsinki University Central Hospital, where the isolation of the PBMC was also performed. The cells (20.000 cells/plate) obtained with each anti K562/PCLP MAb, or with the control MAb (GAD6, anti-glutamate decarboxylase antibodies) using this magnetic bead technique, or unfractionated PBMC were seeded on small cell culture dishes (diameter 5 cm) mixed in a semi-solid methylcellulose medium containing growth factors for CFU (colony forming units)-GM (granulocyte/monocyte) or BFU (burst forming units)-E (erythrocytes). By this technique the first trial (after growth of 14 days) gave the following results:

In CFU-GM plates:

- 3 small colonies (not identifiable by colony morphology) from the cells isolated with MAb BE6 D9 and
- 1 small non-identifiable colony from the non-selected PBMC fraction cells were obtained;
- the other cells did not give raise to any colonies.

In BFU-E plates:

- 1 small colony from MAb BE6 D9 cells
- 3 small colonies from CF9 B8 cells

The results are shown in Figs. 10 and 11, wherein a specific staining of a bead at 100-110 kDa and a specific cell staining was observed.

In Fig. 12 these new MAbs are tested with respect to their binding capacity to recombinant GST (glutathione transferase)-PCLP.

Lane 1: Supernatant of the lysed bacteria making the recombinant GST-PCLP (extracellular domain) fusion protein.
Lane 2: GST-PCLP (extracellular domain) fusion protein purified

by affinity chromatography on gluthathione agaraose.

The apparent MW of the fusion protein is about 80 kDa (expected size).

New monoclonal subclones (undiluted supernatants):
CC9/E9, CF9/B8, BB8/B11 (deposited), BE6/D9
(all recognize the PCLP-fusion protein, the BE6/D9 reaction is the strongest).

Control:
MoCo, mouse monoclonal anti-GAD65 ab (negative control).

For example, BB8 is D1 BB8 BB11 has been made against the K562 cells by IF it stains the cell membrane of K562 cells. It does not stain glomeruli or endothelial cells by IF and it binds to podocalyxin extrapeptide by ELISA. Further, it binds to GST (glutathione transferase)-PCLP extradomain fusion protein by immunoblotting and binds to about 110 and 155 in CFU cells, 110 and 180 in K562 cells and 110 in glomeruli by immunoblotting. Thus, it binds PCLP peptide and stains hematopoietic cells, but does not bind to glomeruli or endothelia at tissue sections. By this exclusion evidence it is functionally specific for hhPCLP.

According to the immunoblotting data the size of hhPCLP may depend on the cell type, but in all cell types the 100-110 kDa band is present. Polyacrylamide gel electrophoresis is not a very accurate technique for the estimation of molecular radius of the proteins. In gels made in successive days the mobility of the proteins is not absolutely identical. So a certain tolerance is needed for the MW determinations. Also the protein extracts made from living cells may contain proteins at various stages of synthesis and proteins degraded by enzymes liberated during the isolation process. These variants have different mobilities in the gels although they all may contain the same antigenic epitope/s. This is inherent to the method, however, the functional specificity for hhPCLP was proven with the aforementioned staining methods, especially the stainings with BB8 antibody.

BUDAPESTER VERTRAG ÜBER DIE INTERNATIONALE ANERKENNUNG DER HINTERLEGUNG VON MIKROORGANISMEN FÜR DIE ZWECKE VON PATENTVERFAHREN

INTERNATIONALES FORMBLATT

Prof. D. Kerjaschki
Ganglbauergasse 33
1160 Wien
Austria

EMPFANGSBESTÄTIGUNG BEI ERSTHINTERLEGUNG, ausgestellt gemäß Regel 7.1 von der unten angegebenen INTERNATIONALEN HINTERLEGUNGSSTELLE

I. KENNZEICHNUNG DES MIKROORGANISMUS	
Vom HINTERLEGER zugeteiltes Bezugszeichen: DI B8 BB11	Von der INTERNATIONALEN HINTERLEGUNGSSTELLE zugeteilte EINGANGSNUMMER: DSM ACC2544
II. WISSENSCHAFTLICHE BESCHREIBUNG UND/ODER VORGESCHLAGENE TAXONOMISCHE BEZEICHNUNG	
Mit dem unter I. bezeichneten Mikroorganismus wurde ( ) eine wissenschaftliche Beschreibung ( ) eine vorgeschlagene taxonomische Bezeichnung eingereicht. (Zutreffendes ankreuzen).	
III. EINGANG UND ANNAHME	
Diese internationale Hinterlegungsstelle nimmt den unter I bezeichneten Mikroorganismus an, der bei ihr am 2002-05-22 (Datum der Erst-hinterlegung)[1] eingegangen ist.	
IV. EINGANG DES ANTRAGS AUF UMWANDLUNG	
Der unter I bezeichnete Mikroorganismus ist bei dieser Internationalen Hinterlegungsstelle am eingegangen (Datum der Erst-hinterlegung) und ein Antrag auf Umwandlung dieser Ersthinterlegung in eine Hinterlegung gemäß Budapester Vertrag ist am eingegangen (Datum des Eingangs des Antrags auf Umwandlung).	
V. INTERNATIONALE HINTERLEGUNGSSTELLE	
Name: DSMZ-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH Anschrift: Mascheroder Weg 1b D-38124 Braunschweig	Unterschrift(en) der zur Vertretung der internationalen Hinterlegungsstelle befugten Person(en) oder des (der) von ihr ermächtigten Bediensteten: U. Weiss Datum: 2002-06-03

[1] Falls Regel 6.4 Buchstabe d zutrifft, ist dies der Zeitpunkt, zu dem der Status einer internationalen Hinterlegungsstelle erworben worden ist.

CLAIMS

1. A cell surface marker for identifying, purifying, isolating and manufacturing hematopoietic stem cells useful for providing stem cells for treating blood diseases or deficiencies and damages in the blood forming tissues, characterised in, that it is a glycosylated human hematopoietic podocalyxin-like protein (hhPCLP) expressed in human hematopoietic tissues and/or cells said hhPCLP having in its glycosylated form an apparent MW of approximately 100-110 kDa as determined by sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE).

2. The cell surface marker according to claim 1, characterised in, that said hhPCLP has a specific glycosylation pattern distinguishing it from human podocalyxin-like proteins (hPCLPs) expressed by glomerular and endothelial tissues and/or cells as well as podocalyxins (PCLPs) of non-human origin.

3. The cell surface marker according to claim 1, characterised in, that said hhPCLP is recognizable by antibodies capable of specifically binding to PCLP.

4. The cell surface marker according to claim 1, characterised in, that said hhPCLP is recognizable by antibodies capable of specifically binding to epitopes on the extracellular part of PCLP.

5. The use of human hematopoietic tissue derived podocalyxin-like protein (hhPCLP) as a marker for identifying, purifying, isolating and manufacturing hematopoietic stem cells.

6. The use of human hematopoietic tissue derived podocalyxin-like protein (hhPCLP) for providing antibodies having the capacity of specifically recognizing hhPCLP by raising antibodies against the extracellular parts of PCLP, preferably hPCLP, most preferably hhPCLP.

7. The use of the antibodies recognizing extracellular parts of PCLP alone or attached to a solid carrier for identifying, purifying and/or isolating hematopoietic stem cells from blood prod-

ucts.

8. The use according to claim 7, characterised in, that the antibodies are monoclonal or polyclonal antibodies.

9. A method for identifying hematopoietic stem cells from blood products, characterised in, that a blood product is contacted with an antibody capable of specifically binding to PCLP, preferably hPCLP, most preferably hhPCLP.

10. A method for purifying hematopoietic stem cells from blood products, characterised in, that the blood product is contacted with an antibody capable of specifically binding to an extracellular part of PCLP, preferably hPCLP, most preferably hhPCLP.

11. A method for isolating hematopoietic stem cells from blood products, characterised in, that the blood product is contacted with an antibody capable of specifically binding to an extracellular part of PCLP, preferably hPCLP, most preferably hhPCLP.

12. A method for manufacturing hematopoietic stem cells, characterised in, that pluripotent hematopoietic stem cells identified with an antibody specifically binding to an extracellular part of PCLP, preferably hPCLP, most preferably hhPCLP are isolated and optionally cultivated.

13. A method for treating blood diseases, characterised in, that a subject in need is provided with a transplant comprising an effective amount of isolated and optionally cultivated pluripotent hematopoietic stem cells carrying a PCLP-marker.

14. A method for assessing the quality of a stem cell population, characterised in, that it comprises the steps of contacting a population of isolated known stem cell marker positive cells with an antibody capable of specifically binding to PCLP, preferably hPCLP, most preferably hhPCLP and recording the PCLP positivity of said stem cell population.

15. A method according to claim 14, characterised in, that the known stem cell marker is a stem cell marker selected from a

group consisting of CD34+, CD38, CD90 and CD117.

16. An isolated and purified hematopoietic stem cell, characterised in, that it expresses the human hematopoietic podocalyxin-like protein (hhPCLP) according to claim 1 which is present in lysates of said stem cells.

17. The stem cell according to claim 16, characterised in, that the human hematopoietic podocalyxin-like protein expressing cell is a cell of myeloid, erythroid, megacaryoid lineage, a colony forming cell present among CD positive cells in mobilized peripheral blood or a cell from a myelogenous leukemia cell line.

18. The stem cell according to claim 17, characterised in, that the colony forming cell is CFU-GM (colony-forming unit-granulocyte-monocyte), CFU-G (colony-forming unit-granulocyte), CFU-M (colony-forming unit-monocyte) or BFU-E (burst-forming unit-erythroid) cell.

19. The isolated and purified hematopoietic stem cell according to claim 16, characterised in, that the myelogenous leukemia cell line cell is a cell of the K562 leukemia cell line.

20. Preparation or culture of hematopoietic stem cells wherein more than 20%, preferably more than 50%, more preferred more than 70%, especially more than 90%, of the cells are pluripotent hematopoietic stem cells.

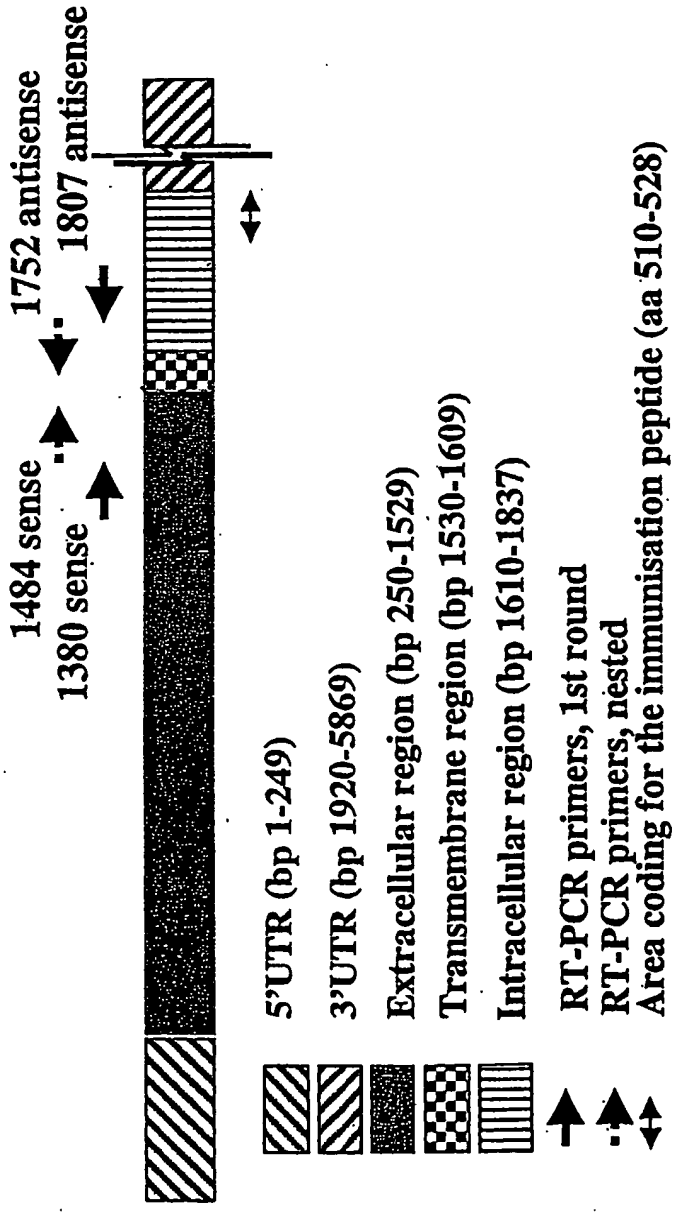
21. Preparation or culture according to claim 20, characterised in that more than 95%, preferably more than 98%, especially about 100%, of the cells are pluripotent hematopoietic stem cells.

22. Preparation or culture according to claim 20 or 21, comprising more than 20%, preferably more than 30%, especially more than 50%, of the cells are hhPCLP positive cells.

23. Preparation or culture according to any one of claims 20 to 22, characterised in that more than 70%, preferably more than 90%, of the cells are hhPCLP positive cells.

24. Monoclonal antibodies being specific for hhPCLP.

25. Antibody according to claim 24, characterised in that it is the antibody DI BB8 BB11, deposited on 21 May 2002 at the DSM2 Braunschweig, Germany, with the deposit number.....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3'UTR (bp 1920-5869)
Extracellular region (bp 250-1529)
Transmembrane region (bp 1530-1609)
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RT-PCR primers, 1st round
RT-PCR primers, nested
Area coding for the immunisation peptide (aa 510-528)

Fig. 1

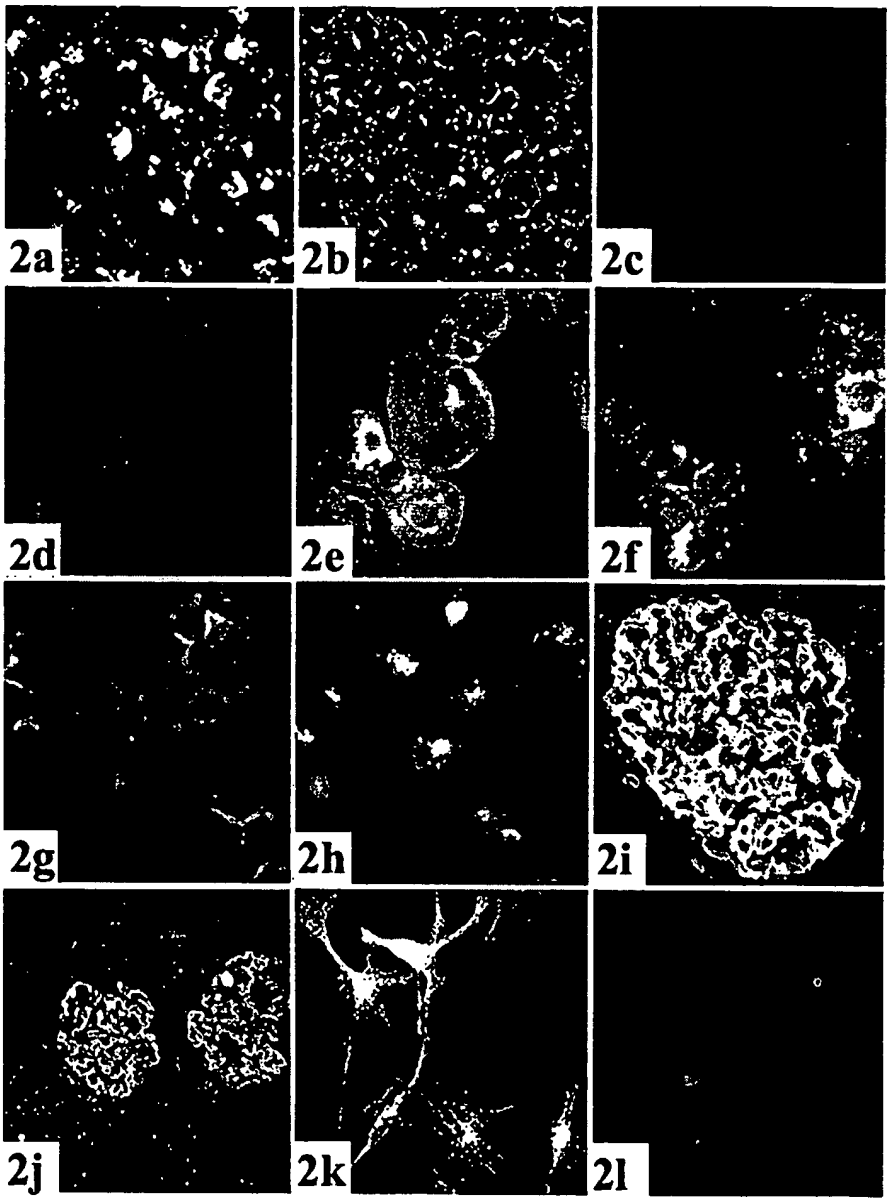

Fig. 2

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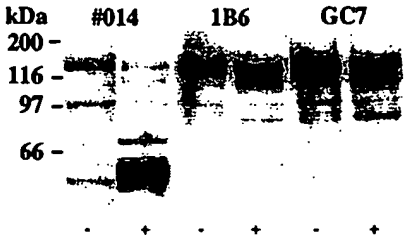
OSGE



3b
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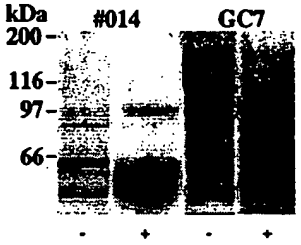
OSGE



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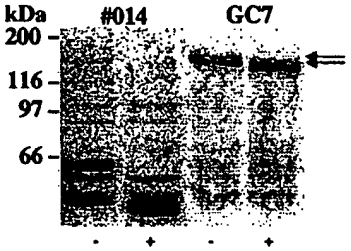
Endo-F



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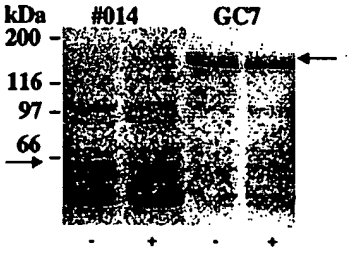

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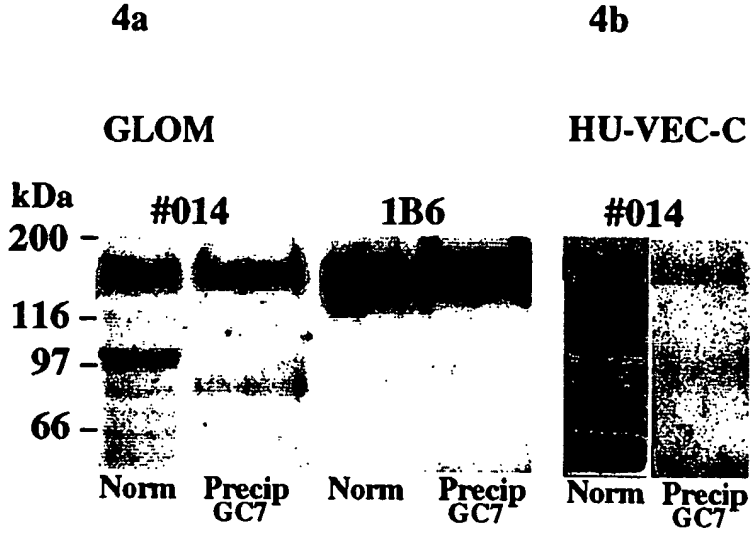

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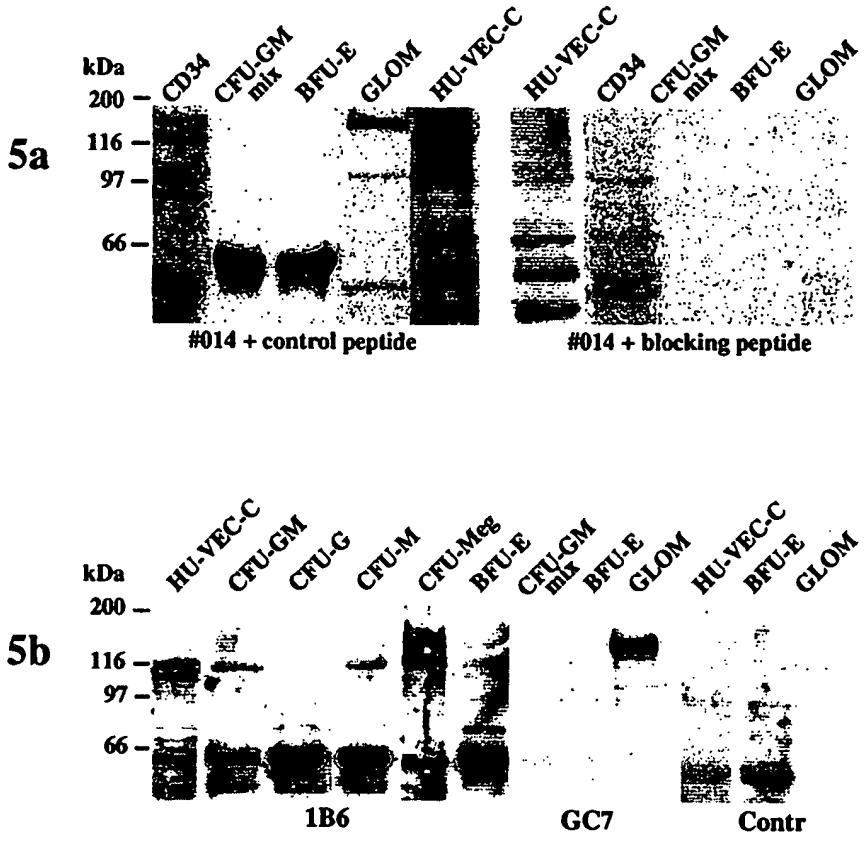
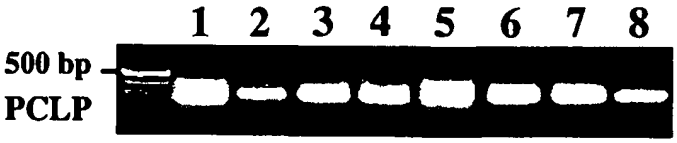


Fig. 5

6a



6b

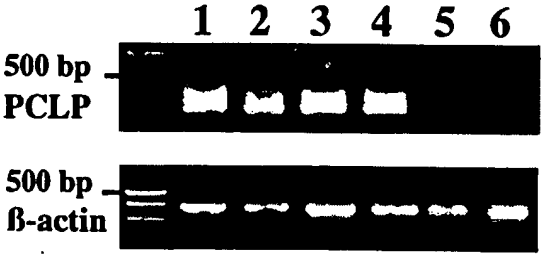


Fig. 6

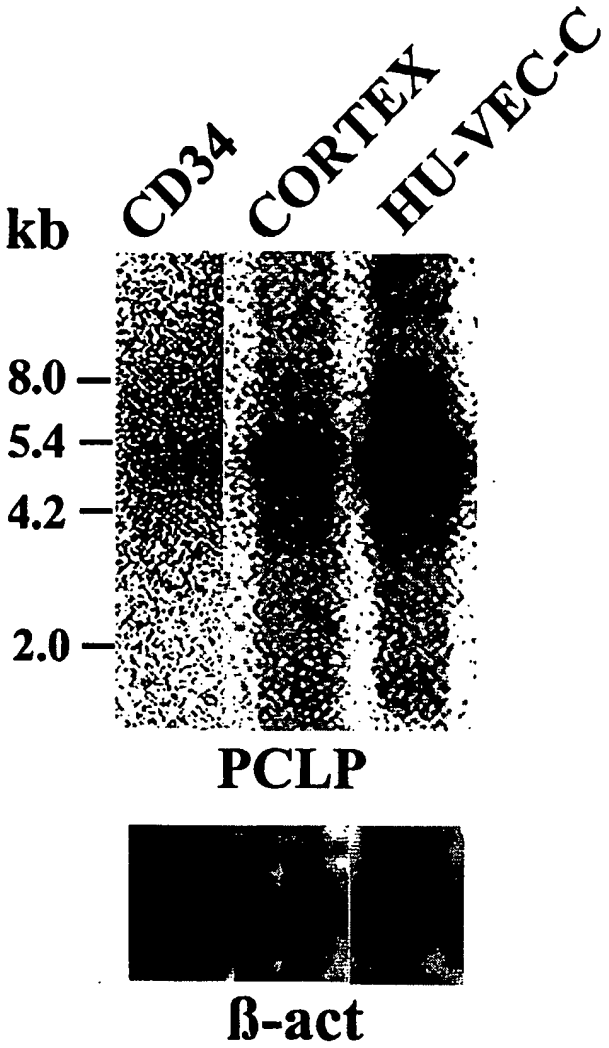


Fig. 7

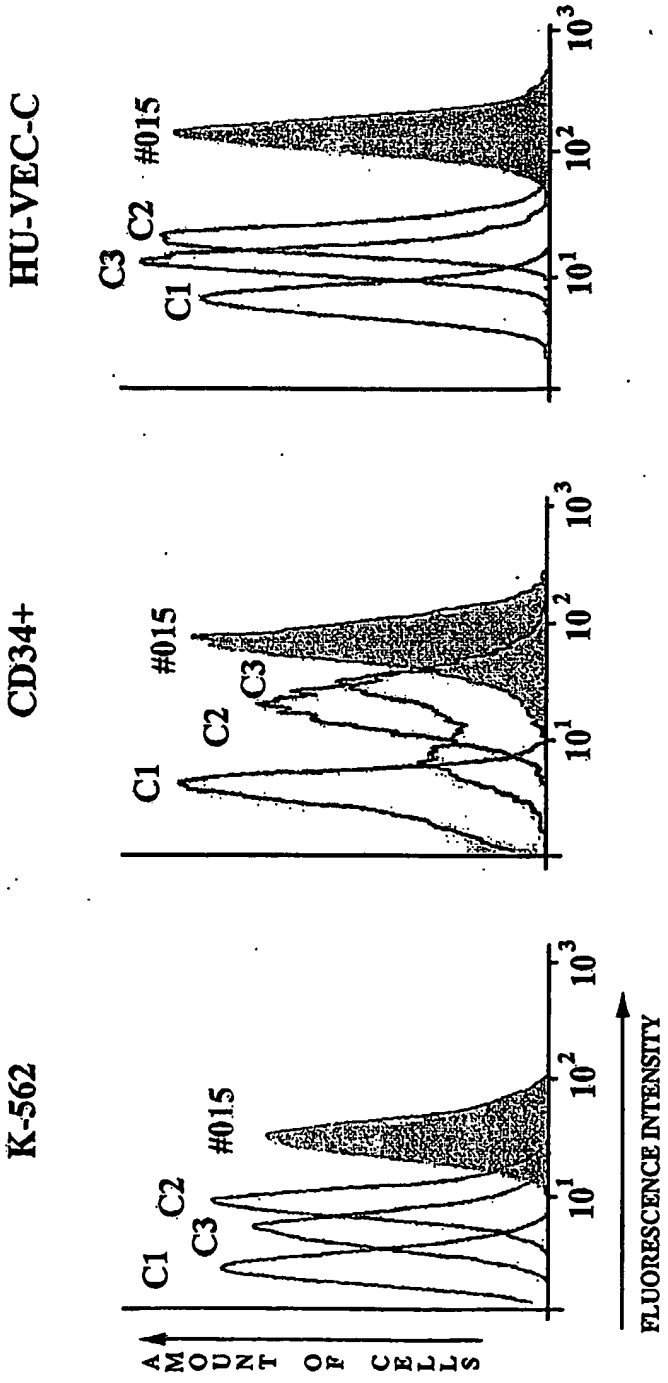


Fig. 8a

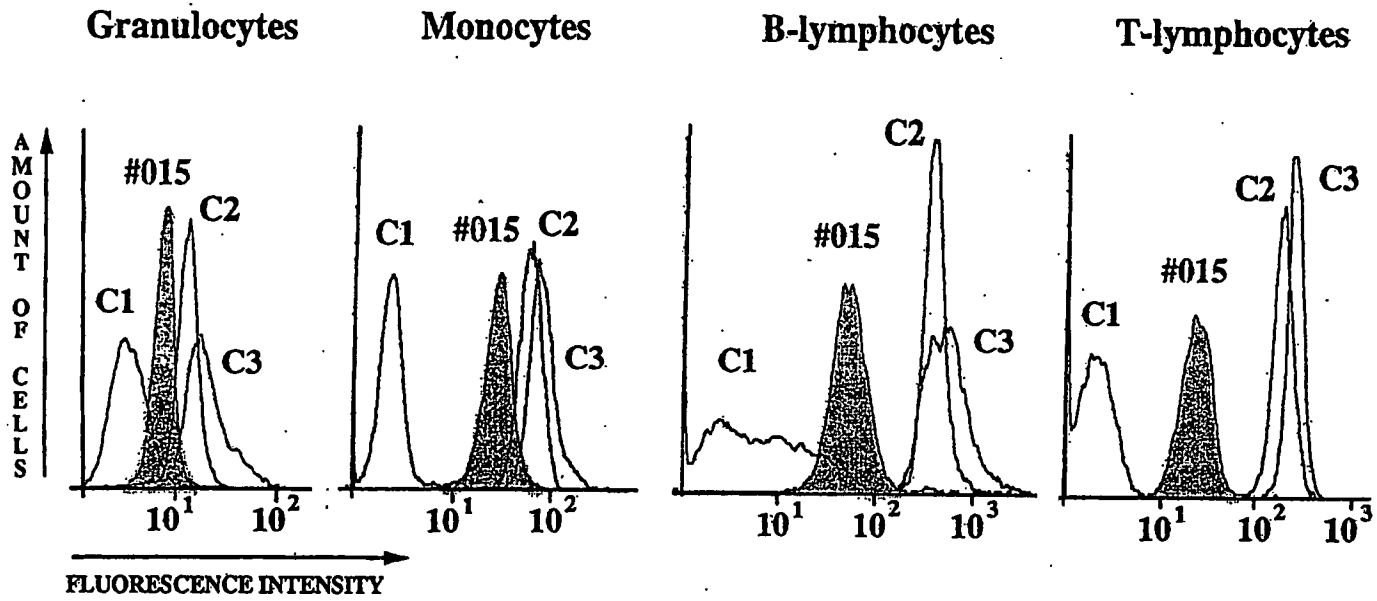
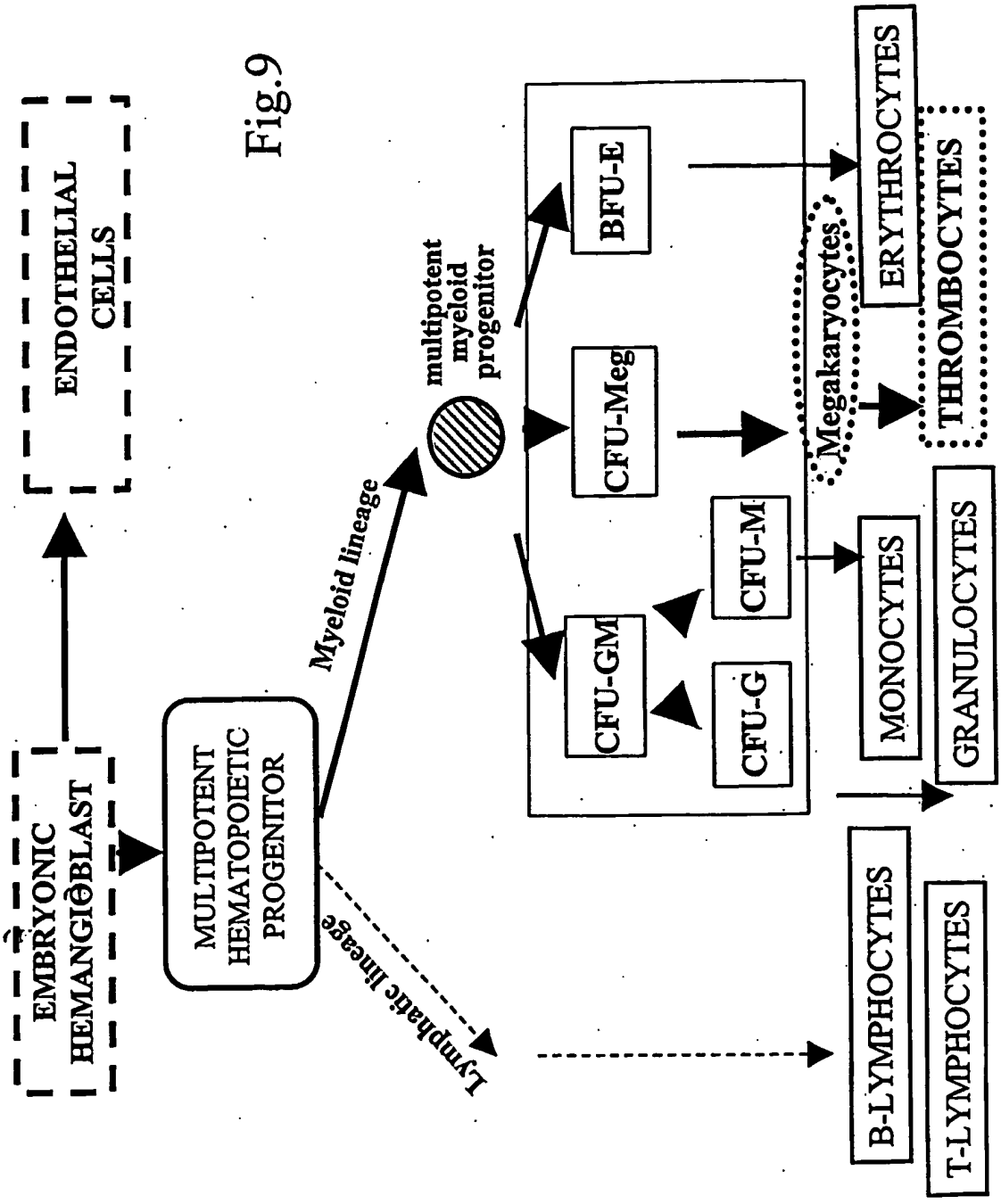


Fig. 8b

Fig.9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ENDOTHELIAL CELLS
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Myeloid lineage
Lymphatic lineage
multipotent myeloid progenitor
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CFU-Meg
CFU-GM
CFU-M
CFU-G
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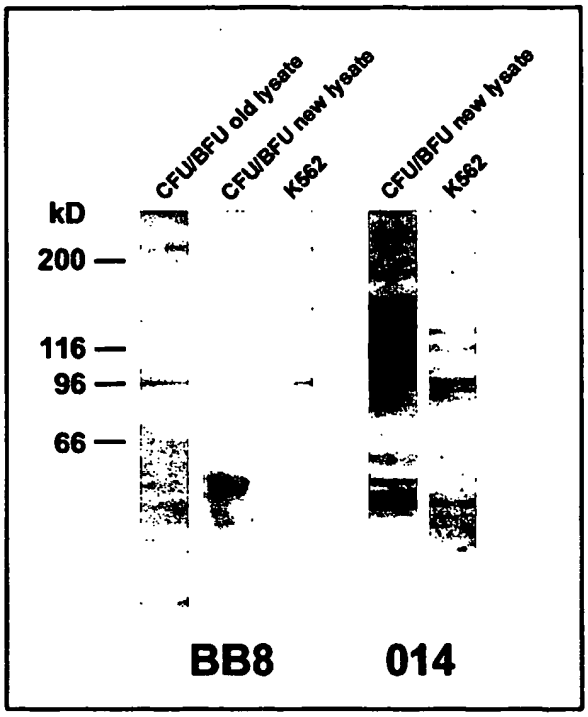
THROMBOCYTES
MONOCYTES
GRANULOCYTES
B-LYMPHOCYTES
T-LYMPHOCYTES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CFU/BFU new lysate
K562
CFU/BFU new lysate
K562
kD
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116
96
66
BB8
014

Fig. 10

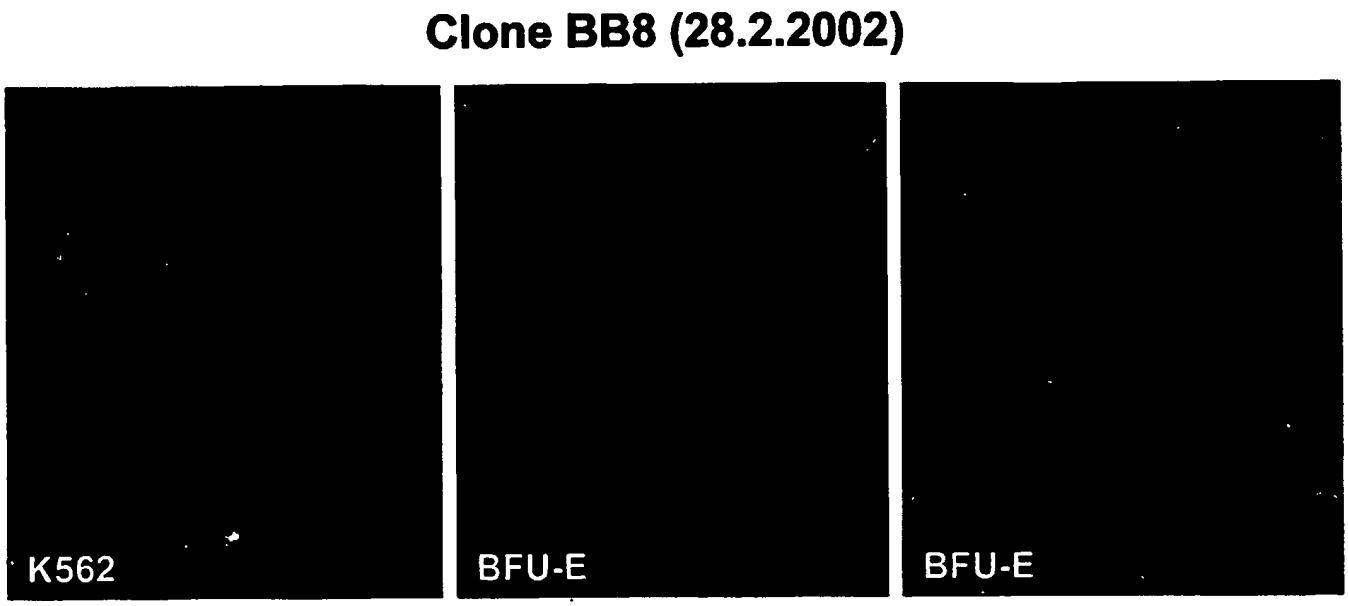
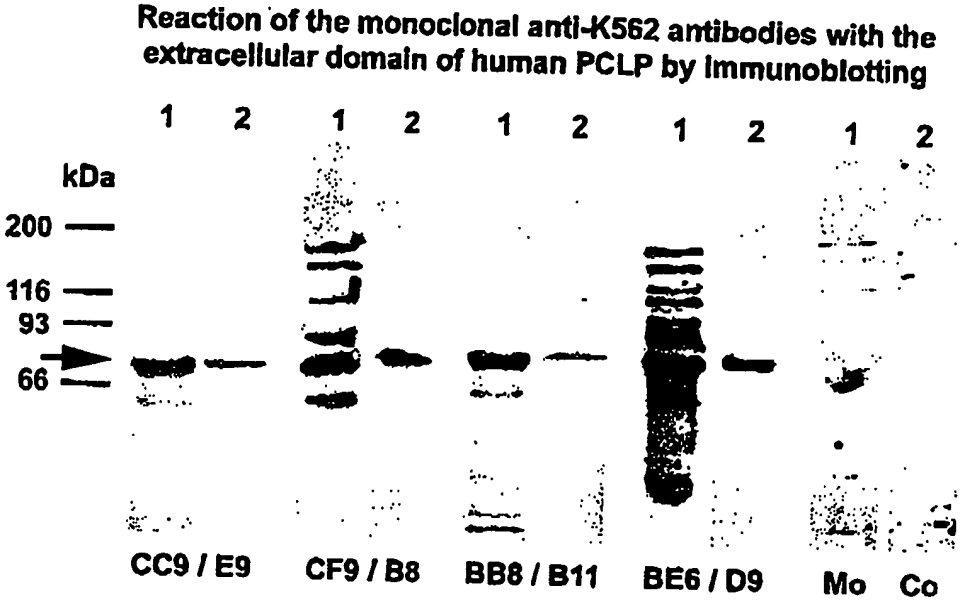


Fig. 11


Reaction of the monoclonal anti-K562 antibodies with the extracellular domain of human PCLP by immunoblotting
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66
CC9 / E9
CF9 / B8
BB8 / B11
BE6 / D9
Mo
Co

Fig. 12

SEQUENCE LISTING

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Thr His Leu

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Glu Leu Lys

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